THE FINE STRUCTURE OF LEUCOCYTES

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1964

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--with special reference to pyrogen production

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine at Yale University School of Medicine, New Haven, Connecticut. April 1, 1968

To Kay

The author wishes to express his gratitude for the invaluable advice of his three research advisors, Dr. Russell Barrnett, Dr. Elisha Atkins, and Dr. Phyllis Bodel.

Table of Contents

INTRODUCTION

The participation of leucocytes in host response to various diseases has been long recognized. Many illnesses have characteristic alterations in the number of different types of circulating leucocytes. In stained tissue section, the presence of certain kinds of leucocyte is helpful in establishing a diagnosis. Clinical observations of the critical role played by the leucocyte in health and disease have led to much recent research in the functions of the different morphologic types of leucocyte.

This study has focused on one function of leucocytes, the production of substances which mediate febrile response. Only cells which are known to release such pyrogenic substances have been studied: the polymorphonuclear leucocyte, the monocyte, and the alveolar mononuclear cell.

Leucocyte ultrastructure

Though many reports on the ultrastructure of leucocytes have appeared in the literature (25,26,27,28,29,69,75,81,99,100,110,111, 115,126,127,138,166,168,171), technical limitations of early electron microscopy and difficulties with achieving reproducible fixation, especially with the polymorphonuclear (PMN) leucocyte, have produced disappointing results until recently. Anderson (2) utilized glutaraldehyde and osmium fixed preparations in a study of the normal human leucocyte. The normal PMN leucocyte characteristically possessed several nuclear lobes in each section, with chromatin material peripherally distributed in relation to the nuclear membrane. The cytoplasm contained the typical granules (lysosomes), which were neutrophilic in Wright's stained blood smears. These granules were round to oval, approximately 0.5 micra in diameter, were seen in a wide

range of electron densities, and were surrounded by a membrane which only occasionally could be delineated. The cytoplasm also contained dense angular deposits of particulate glycogen (200-250 A^{o} diameter), occasional short pieces of rough endoplasmic reticulum, a perinuclear Golgi region, and many types of vesicles.

The monocyte, with its nucleus which often appeared U-shaped in thin section, contained a variable number of dense lysosomes of slightly smaller size than the PMN granules (lysosomes). Also characteristic of the monocyte was an extensive Golgi region, many mitochondria, abundant free ribosomes, short strands of rough endoplasmic reticulum, and occasional microtubules. The fine structure of the other cells occurring in normal human blood was also described (2).

Various tissue macrophages have also been the subject of many electron microscope investigations, but the most pertinent for the purpose of the present study was the description of the mouse alveolar macrophage (97). This large cell, with many cytoplasmic processes, contained an abundance of lysosomes of many shapes and sizes, much endoplasmic reticulum, a well developed Golgi zone, and many mitochondria.

Leucocyte lysosomes

A fine structural feature common to the three cells described, all of which have significant phagocytic capability, is the abundance of electron-dense lysosomes. Lysosomes are known to play an integral role in the process of intracellular digestion, to be described later, and qualify for their designation as lysosome on the basis of their content of hydrolytic enzymes. Novikoff et al presented autoradio-

graphic evidence that lysosomes were a product of the Golgi apparatus (123) and this was confirmed by Cohn (52).

The nature of the granule (lysosome) of PMN leucocytes has received considerable interest in recent years. Most electron microscopic, histochemical, and biochemical observations of PMN leucocytic granules have utilized rabbit peritoneal exudate cells. The rabbit PMN leucocyte contains granules which are morphologically different from those of the human, and they are designated as heterophils rather than neutrophils on the basis of their greater affinity for acidic or basic components of Wright's stain.

Two laboratories have presented evidence that the rabbit PMN leucocyte contains granules of three morphologic types: a dense, osmophilic type, a uniformly less dense type, and a "target" type with dark center and lucent periphery (106,175). Bainton and Farquhar have shown that the denser granules, which they term azurophilic, are formed exclusively in the progranulocyte stage of maturation from the concave face of the Golgi apparatus, and account for 10-20% of mature PMN granules. The less dense, or specific granules, originate in the myelocyte stage of development from the convex surface of the Golgi apparatus, and comprise 80-90% of mature PMN granules. No description of "target" granules was made (11).

Histochemical determinations have demonstrated that all granules possess nonspecific alkaline phosphatase, and all but some intermediate-sized, low density granules possess nonspecific acid phosphatase (86,87,170). Biochemical anlaysis of the lysosomal fraction of homogenized rabbit PMN leucocytes revealed the presence of acid and al-

kaline phosphatase, B-glucuronidase, acid ribonuclease, acid DNAse, arylsulphatase, peroxidase, B-galactosidase, acid cathepsins, phagocytin, lysosyme, lipase, and phospholipase (53,64). The proteolytic activity of rabbit PMN leucocyte lysosomes was found to be attributable to cathepsins A, D, and/or E, but not to B and C (165). Light microscopic histochemical studies of PMN leucocytes (61,173) and other phagocytic cells have confirmed the biochemical findings of hydrolytic granule enzymes.

PMN leucocyte lysosomal membranes had antigenic similarity to erythrocyte membranes (131). Intact lysosomes *in vivo* and *in vitro* were rendered more fragile to spontaneous release of their enzymes by exposure to endotoxin, etiocholanolone, vitamin A, and streptolysin S, while they were stabilized by exposure to cortisone or chloroquine (169).

The Process of phagocytosis

Phagocytosis is an important function in PMN leucocytes, monocytes, and macrophages, and is the mechanism by which sequestration and ultimate digestion of extracellular particulate matter occurs *in vivo*. Phagocytosis initiates a number of morphological, physiological, and biochemical changes which have been the subject of much recent research.

The initial step in phagocytosis by PMN leucocytes appeared to be an interaction between the cell membrane and the object to be ingested (35). Loni *et al* found that phagocytosis of ferritin by HeLa cells was slow, but after addition of ATP to the medium, the rate was markedly enhanced (109). However, North showed that ATPase activity, although confined to the cell membrane, did not show an increase at the site of attachment with the object (122). The PMN leucocyte could ingest many different particles in the absence of homologous serum, but

efficient phagocytoses of certain particles such as bacteria appeared to require serum (37).

Once the extracellular material has been incorporated into a phagocytic vacuole by engulfment, intracellular digestion, using the hydrolytic enzyme complement of lysosomes, begins. As originally described by deDuve (62), lysosomes were thought to serve primarily as "suicide bags" in post-mortem cell autolysis or in digestion of out-dated cell organelles.

However, in phagocytic cells, it appears that lysosomes participate extensively in the process of regulated digestion of phagocytosed material. Bensch, Gordon, and Miller were able to label DNA-protein coacervates with colloidal gold and trace the natural history of phagocytosed coacervates in cultured "L" strain fibroblasts (19,20). The labeled coacervates were phagocytosed by engulfment, and the resulting membrane-bound phagocytic vacuole was seen to fuse with lysosomes containing acid phosphatase and other enzymes. The fluid within the vacuoles was seen to be rapidly absorbed, and more lysosomes continued to fuse with the digestive vacuole. At first, acid phosphatase activity was seen to be located at the periphery of the vacuole, and later was uniformly distributed as the density of the vacuole decreased. The digestive vacuoles seemed to evolve into multivesicular bodies and dense bodies, all of which contained acid phosphatase and esterase activities. Throughout the process, coalescence of two digestive vacuoles could be observed, but discharge into the extracellular medium was only rarely seen. The "end product" dense body coalesced with other dense bodies and with digestive vacuoles. Strauss (155)

performed a similar experiment using the light microscope demonstrating coalescence of lysosomes with phagocytic vacuoles containing horseradish peroxidase.

Similar studies have been performed on polymorphonuclear cells obtained from peritoneal exudate. Hirsch, using phase contrast cinemicrophotography, was able to demonstrate the coalescence of lysosomes and phagocytic vacuoles in the living phagocytic PMN leucocyte (84). Spitznagel and Chi showed that fast green-positive lysosomal basic protein coated phagocytosed bacteria within newly formed digestive vacuoles (151). Electron micrographs published from work of different laboratories document the event of granule-phagocytic vacuole coalescence with subsequent digestion of phagocytosed material (106,107, 118,175). The eventual appearance of bacterial breakdown products in the medium has been shown biochemically (45).

Pinocytosis, a process similar to phagocytosis, in which very small vesicles containing extracellular fluid are pinched off from the limiting membrane of the cell, was studied by Cohn in cultured mouse peritoneal macrophages (52,56,58). Pinocytic vesicles behaved much like phagocytic vacuoles, merging with one another and with preformed lysosomes (52).

Physiological variables which have bearing on phagocytosis include motility and adhesiveness. During phagocytosis <u>in vitro</u> PMN leucocytes demonstrated a decreased motility because of stickiness to one another and to "intrinsic factors" (46). This increased stickiness occurred in cells which were not actively phagocytosing, as well as in those which were (108). PMN leucocytic aggregation required the presence

of divalent cations (46,102). However, adhesiveness did not appear necessary for phagocytosis, since under hyperosmolar conditions, adhesiveness disappeared but phagocytosis did not (103). In macrophages, attachment of particles was a linear function of the particle load, but the percent ingestion decreased as the load increased (132). Attachment in macrophages did not seem to require divalent cations, but ingestion required both serum and divalent cations (132).

The Biochemistry of Phagocytosis

Two excellent reviews of the biochemistry of leucocytes (43) and of phagocytic cells (95) have been published recently. The amount of research in this area is evidenced by a combined total of 1000 cited articles. This paper will present selected aspects of metabolism of phagocytosis that seem relevant to the area of investigation.

Leucocyte oxygen consumption increased following phagocytosis, and the stimulation of respiration was proportional to the load of particles ingested within a finite range (94). The major pathway for energy production in most phagocytic cells was glycolysis (13), and phagocytosis *in vitro* proceeded anaerobically (94). The hexose monophosphate (HMP) shunt, respiration, and oxidative phosphorylation were less important pathways yielding energy (30,98). Respiration, however, was necessary for efficient phagocytosis in the alveolar macrophage (125). Respiratory rates differed amont the phagocytic cells in the resting state, and the order of decreasing oxygen utilization was alveolar macrophage, monocyte, and PMN leucocyte (163). Oxygen consumption *via* the HMP shunt was greatly increased during phagocytosis, as was glycolysis (21,89,94). The source of PMN leucocyte glucose was

probably glycogen (12), and histochemical studies have demonstrated abundant stores in those cells (164), but Dalquist has shown that all PAS-positive material within the cells was not glycogen (60). Glycolysis, while apparently required for phagocytosis, was not required for intracellular degradation of ingested material (46).

More recently, it has been shown that the increased respiration during phagocytosis began before noticeable degranulation (174). Also, increased respiration could be partially blocked without a decrease in phagocytosis by selected concentrations of colchicine (113). These results, and those of other drug-induced metabolic blockades (37,42,43) suggested that phagocytosis and certain metabolic changes normally found to accompany it could be dissociated.

Recent interest in incorporation of precursors of phospholipid during phagocytosis (96,124,143) have made possible quantitative estimates of membrane utilization which suggested that there was a specific increase in membrane synthesis during phagocytosis (21). Cline has demonstrated an increased turnover of RNA and an increased uptake of RNA precursors from the medium during phagocytosis which were proportional to the number of ingested particles (41,42). Graham et al exposed guinea pig PMN leucocytes to endotoxin, deoxycholate, and digitonin, finding that these agents produced metabolic changes identical to those seen during phagocytosis, though no phagocytosis was observed morphologically (72).

Macrophages and monocytes

The origin and differentiation of tissue macrophages has been extensively studied. Bennett has pointed out that macrophages of various sites were not homogeneous; lung macrophages were especially

different by virtue of a high mitotic rate, dependence on aerobic metabolism and pyroninophilic cytoplasm (16). Two-thirds of lung macrophages appeared to originate as blood monocytes; the other third appeared to come from tissue mesenchyme (16). Cohn et al studied the in vitro differentiation of peritoneal macrophages, finding that differentiation included an increase in cell size and an accumulation of acid phosphatase-positive, phase-dense granules, mitochondria, and lipid stores (48,54). Pinocytosis played a major role in macrophage differentiation (50,51), and endotoxin and BCG appeared to increase the rate of differentiation (54).

The blood monocyte, generally thought to be an immature member of the reticuloendothelial system (67) has proved a difficult cell to investigate. Until recently, it could not be obtained in sufficiently pure preparations. However, a method for isolation based on its lower density from other leucocytes, and a description of differentiation into macrophages, has recently been published (18). Endotoxins increased the rates of phagocytosis and pinocytosis in monocytes (18,55,137).

The PMN leucocyte in inflammation

The physiological role of the PMN leucocyte in inflammation involves many functions including diapedesis from blood vessels (73), chemotaxis (139), phagocytosis, and release of various humoral factors. Amont these humoral factors, released cathepsins could be found in Shwartzmann (80,159) and Arthus reactions (39,59,119,165), and appeared to damage vascular basement membrane (44). PMN leucocytes also released a "delayed permeability factor" (117,119), kinin-forming and degrading enzymes (74), mast-cell rupturing substance (90,144), a

substance triggering a delayed inflammatory response (117), and an endogenous pyrogen.

Endogenous pyrogen (EP)

The existence of an endogenous pyrogen (EP) clearly distinct from the pyrogen of Gram-negative bacterial origin, lipopolysaccharide endotoxin, was first recognized by Bennett and Beeson (17) in 1953. Since that time, EP has been shown to play an essential role in the pathogenesis of many experimental and some clinical fevers (10). Much subsequent evidence indicates that the production of fever by many microbial and some nonmicrobial agents is mediated through a final common pathway of one or more EPs, released by cells of the host.

Until recently, the only cell definitely implicated in EP release was the PMN leucocyte, but the rabbit tissue mononuclear cell of respiratory passages, spleen, and lymph nodes (6,78) and the human blood monocyte (32) are now confirmed as additional sources of endogenous pyrogen. In addition, Atkins and Snell have found a tissue pyrogen extractable from normal rabbit tissues which does not appear to arise from endotoxin or PMN leucocyte contamination (149).

Substances capable of stimulating normal cells to release EP are termed activators, for the normal cell incubated in saline without activator produces no EP. Among the known activators are endotoxin (150), myxovirus (6), cryptococci and their derivatives (79), tuberculin (8), staphylococci and their products (9), and etiocholanolone (33). Cells from inflammatory exudates are already activated, and release EP in potassium-free saline without further stimulation (22).

Several attempts to characterize EP chemically have met with

some success. PMN leucocyte pyrogen derived from sterile peritoneal exudates appears to be a lipid-protein complex with a molecular weight between 10,000 and 20,000, and an isoelectric point about pH 8 (68,133). Similarly, a virus-induced EP from rabbit serum appears to be a protein (7

Release of EP by PMN leucocytes is initiated by phagocytosis, but only after a definite lag period. Berlin and Wood studied the effects of metabolic inhibitors on EP release by PMN leucocytes from rabbit peritoneal exudate. They found that release of EP is independent of the major pathway of energy production by PMN leucocytes, glycolysis, since it is not blocked by sodium fluoride. EP release may be dependent on a sulfhydryl-containing enzyme since it is blocked by arsenite (24). Murphy, however, found little inhibition by arsenite (120). Pyrogen release in peritoneal exudate leucocytes is strongly inhibited by physiological extra-cellular concentrations of potassium ion; this inhibition is potentiated by calcium ion. Ouabain counteracts the inhibitory effect of these cations, so it is thought that potassium inhibition of EP release may set through membrane transport of potassium. The release of EP resembles lysosyme and aldolase release by PMN leucocytes, which is similarly blocked by arsenite and potassium (23).

Differential centrifugation of disrupted PMN leucocytes by Herion et al (83) showed that the greatest pyrogenic activity per gram of protein was found in the lysosome fraction, lesser activity in the 8000Xg supernatant, and least in the 400Xg sediment. Lysosomes from normal rabbit PMN leucocytes produced the same degree of fever as those from peritoneal exudate cells; normal human PMN leucocytes pro-

duced no fever. Tanaka, using more elaborate centrifugations, found the pyrogenic activity (tested in endotoxin-tolerant recipients) of endotoxin-activated PMN leucocytes concentrated in the lysosomal and small particle fractions, but not in nuclear, ribosomal, or soluble fractions (157). Wood et al have attempted to confirm these results without success (5).

Goodale, Hillman, and Fillmore have published two major papers in which they describe electron microscopic observations of normal and endotoxin-activated human blood leucocytes, and those occurring in patients with fever (70,71). In activated cells, a finely granular extracellular material was consistently seen, but was absent from most controls. Such material was also present in two preparations activated by virulent pneumococci, and in some specimens from patients with naturally occurring fevers of various etiologies. In one cell from a patient, this granular material was found in a digestive vacuole. Chemically purified EP dried on a grid is said to resemble the amorphous material in basic structure, and the authors have tentatively identified the amorphous granular material as EP (70,71). Lockwood and Allison report the existence of material resembling the amorphous granular substance in digestive granules of rabbit peritoneal exudate preparations (106). However, Graham et al found no morphologic changes on exposure of guinea pig PMN leucocytes from peritoneal exudate to endotoxin (72).

Purpose of this study

The object of this investigation was to determine whether there are characteristic fine structural events associated with the production and release of endogenous pyrogen. It seemed reasonable to confine this study to those cells known to release EP and those activating

agents which had been previously studied in detail. Thus, the focus of the study was on the PMN leucocyte, monocyte, and lung mononuclear cell.

Because of the ubiquitousness of endotoxin as a contaminant of laboratory apparatus, "normal" cells in previously published micrographs had to be held suspect of being unintentionally activated. In each experiment, therefore, a control group was prepared in parallel with the activated preparation using the same reagents and conditions of incubation, but without the activating agent. Assays for EP were preformed to insure that control cells were not activated, and conversly, that EP was present when cells were incubated with the activators. Micrographs of control and activated cells could thus be compared, with confidence that any differences could be attributed to the effects of the activating agent alone.

Since each activating agent could be expected to produce effects on the cell unrelated to EP production and release, it was felt that several different agents should be studied. On the assumption that various activators mobilize EP through similar mechanisms, then effects not found to be common to all the agents could be eliminated from consideration. Conversely, those effects found repeatedly would be more likely to be related to EP production and release. The activation systems chosen were: 1) the rabbit peritoneal exudate PMN leucocyte (using the normal blood PMN leucocyte as a control); 2) the human PMN leucocyte incubated with heat-killed staphylococci; 3) the human PMN leucocyte and monocyte incubated with etiocholanolone; and 4) the BCG-sensitized rabbit lung mononuclear cell incubated with tuberculin.

Viable, virulent bacteria and endotoxin were felt to be too toxic to cells, and therefore likely to produce artefacts related to cell death, so these agents were not used.

Tissue preparation for electron microscopy generally followed standard schedules, altered to some degree for single cells (leucocytes) harvested by centrifugation. In addition to the classic osmium tetroxide preparations, phosphotungstic acid (PTA) preparations were used to stain basic proteins such as the histones of DNA and lysosome contents (146). Also, since EP is a basic protein (68), either EP or one of its precursors might be expected to stain with PTA.

After preliminary results were analyzed, it became clear that all activating agents produced a multitude of changes in leucocytes. Metabolic inhibition of EP production was therefore used in an attempt to ascertain which morphologic changes were specifically related to mobilization of EP. Bodel has studied the inhibitory effect of 5 micrograms/ml. actinomycin-D on EP release by human leucocytes that have been activated with staphylococci (31). She found that EP release was only partially inhibited if actinomycin-D was added one hour after the addition of staphylococci to cell suspensions. If actinomycin-D was added before that time, no EP was released. By one hour, as can be shown by light microscopy, the PMN leucocytes have phagocytosed many staphylococci. Actinomycin-D has been shown not to alter the process of phagocytosis or the increased cellular utilization of the HMP shunt pathway within this time interval (31,41,42).

Thus, the use in this study of actinomycin-D at 30 minutes following the addition of staphylococci to cell suspensions, a time when there is already significant phagocytosis, ensured that blockade

of EP production and release had occurred. The intra-cellular events related to phagocytosis which proceeds normally could be separated from those related to EP release, which is blocked. This experiment was performed and the preparations processed for electron microscopy.

Biological effects of actinomycin-D

The mechanism by which actinomycin-D blocks release of EP is not known, and it can only be surmised that it is related to its well-known inhibition of RNA synthesis. Two reviews of the effects of actinomycin-D on cells have appeared (134,141). RNA synthesis is completely blocked by high doses of actinomycin-D, but moderate doses selectively inhibit the synthesis of nucleolar RNA (153), itself the obligatory precursor of ribosomal RNA (128,129).

Electron microscopic examinations of many tissues of different species have demonstrated few changes following treatment with actinomycin-D. Examinations of rat liver and HeLa cells treated with actinomycin-D reveal consistent effects limited to the nucleolus which becomes smaller and more homogeneous (92,136,154). In HeLa cells, higher concentrations of actinomycin-D also cause blebs of cytoplasm to be released from the cell into the external medium (92). Nuclei of Rana enbryos contain 200 A^{o} diameter threads after actinomycin-D treatment (91). The effects of actinomycin-D on PMN leucocytes, monocytes, and lung mononuclear cells have not been reported to date.

MATERIALS AND METHODS

General

3-6 kilogram male and female albino rabbits were used both as donors for rabbit blood cells and lung macrophages, and as recipients for pyrogen testing. Healthy adult male volunteers donated human blood. In all experiments, pyrogen-free glassware was prepared and used according to the methods described by Bennett and Beeson (17). Aseptic pyrogen-free tissue preparation techniques were strictly followed in all steps before cell fixation.

Tissue purification

50 ml. rabbit blood was drawn into a heparinized syringe by transthoracic cardiac puncture and was processed by one of three methods to obtain a satisfactory yield of leucocytes. In the first of these, low-speed centrifucation (1200 r.p.m. in an International Refrigerated Centrifuge) at 10° C. was followed by aspiration of the buffy coat (1) and resuspension in normal saline. This technique usually resulted in excessive erythrocytic contamination. The second method, preparation of the buffy coat of rabbit blood in capillary microhematocrit tubes was usually injurious to white cell morphology. The best method, and the one most often employed for both rabbit and human blood, was dextran sedimentation. Two parts 3% dextran was added to one part whole blood immediately following phelebotomy and the mixture was allowed to stand at room temperature for 20-30 minutes in a graduated cylinder. Low-speed centrifugation at 10° C. of the supernatant produced good yields of leucocytes (2×10^8 cells from 50 cc. blood). Moderate erythrocytic contamination was eliminated by adding 3 ml. iced distilled water with agitation for 20 seconds,

followed by 1 m. 3.6% sodium chloride to restore isotonicity, and 5 ml. Krebs-Ringer-Phosphate (KRP) buffer (65). This procedure disrupts red cells but does not appear to affect leucocyte function.

Sterile shellfish glycogen-induced peritoneal exudates in rabbits were prepared and harvested according to the method of Wood (93) after four hours. The exudate was washed with saline and centrifuged.

Preparations of rabbit lung macrophages were obtained, according to the method of Atkins, Bodel, and Francis (6), by three saline washings of the tracheobronchial tree. Immediate centrifugation at 2000 r.p.m. and 10^{o} C. produced yields averaging 10^{9} cells.

Fresh human blood from phlebotomy was processed according to the dextran sedimentation method described earlier.

In vitro experimentation

a) Etiocholanolone

Purified suspensions of human leucocytes (4×10^{7} per ml.) in a medium containing 12% autologous serum, 6000 units penicillin, and 150 mg% glucose in KRP buffer were divided into two groups of flasks. To half the flasks, etiocholanolone in KRP buffer was added to a final concentration of 23 micrograms per ml; to the other half, an equivalent volume of KRP buffer was added. The flasks were incubated at 37^{o} C. for five hours on a Dubonoff apparatus.

b) staphylococci and actinomycin-D

Purified human leucocytes (4×10^{7}/ml.) in a medium containing 15% autologous serum in KRP buffer were divided into four groups of flasks. Heat-killed staphylococci in saline in the ratio 10 per leucocyte were added to groups 1 and 3; groups 2 and 4 received equivalent volumes of normal saline. These groups were then incubated for

30 minutes at 37° C. Groups 1 and 2 were transferred to new flasks containing actinomycin-D (dried on the walls of the flask from acetone solution); the final amount of drug after addition of the cell suspension was 5 gamma/ml of solution. Groups 3 and 4 were merely transferred to clean flasks. Incubation of the four groups (actinomycin plus staphylococci, actinomycin alone, staphylococci alone, and control cells alone) at 37° C. continued for four hours. Samples were processed for electron microscopy at this time, and the remainders were incubated for an additional 18 hours after addition of penicillin and glucose in the concentrations specified above. Pyrogen testing was performed on all supernatants at that time.

c) old tuberculin

Suspensions of cells from lung washings of rabbits sensitized with BCG at least 12 days prior to sacrifice were placed in a medium containing 15% autologous serum, penicillin, and glucose in KRP buffer and divided into two groups of flasks. Old tuberculin in saline was added to one group; the other group received only an equivalent volume of normal saline. Incubation at 37° C. followed for four hours. Supernatants from incubations were routinely tested for pyrogenicity in all but one case by injection into normal rabbits. However, no adequate preparations of incubated lung cells were obtained. Due to unknown technical factors, the cells showed evidence of recent osmotic disquilibrium and/or death.

Electron microscopy

Cells from purifications and incubations were washed once in KRP buffer and centrifuged at low speed for 15 minutes at 37° C. The pellet was then either fixed *in situ* with 3% glutaraldehyde (140) in

a 0.1M sodium cacodylate buffer, pH 7.2, or resuspended in KRP buffer, added to an equal volume of 6% glutaraldehyde in KRP, and recentrifuged. Fixation proceeded for 10-15 minutes for rabbit leucocytes, 15-30 minutes for human leucocytes, and 30 minutes to 1 hour for lung cells. The pellets were washed overnight at 4^{o} C. in 0.1M cacodylate or KRP buffer, and diced into 1 mm^3 pieces.

Pellets were usually refixed in 1% OsO_4 in veronal acetate buffer for 5-15 minutes (leucocytes) or 30-45 minutes (lung cells), followed by dehydration in a graded series of ethanol. After pellets were dealcoholized in propylene oxide, they were embedded in Epon 812 (112), Maraglas (152), or an Epon-Araldite mixture (116). Phosphotungstic acid (PTA) staining was performed on glutaraldehyde fixed pellets by immersion in a solution of 1% PTA in absolute ethanol for 90 minutes after the first change of 100% ethanol during dehydration. This was followed by two 100% ethanol washes and routine embedment. These sections of plastic embedded material, cut on an LKB ultramicrotome, were viewed with a Hitachi HU 11-B electron microscope. Staining of some sections in 1% lead citrate (136) from 30 seconds to 5 minutes or saturated aqueous uranyl acetate (167) was performed occasionally to enhance contrast of cells fixed both in glutaraldehyde and osmium tetroxide.

RESULTS

Normal Human Leucocytes

The appearance of human peripheral blood leucocytes fixed with glutaraldehyde and osmium tetroxide has been investigated by Anderson,[1] and a brief summary of his results has been presented in the Introduction. In this study, however, dextran sedimentation rather than centrifugation was used to obtain pure preparations of leucocytes, and fixation times were considerably shorter than those used by Anderson and others. Therefore, examples of micrographs taken of the commonest types of leucocyte will be presented for comparison with previous studies.

The monocyte (Figure 1) usually had an irregular, convoluted border though its general shape was ovoid. The cell surface was thrown up into many pseudopodia and cytoplasmic veils. The nucleus, with its characteristic central indentation, often was sectioned so as to appear bilobed. The nuclear envelope, actually composed of two closely apposed, parallel membranes, enclosed the peripherally arranged chromatin material, the nucleolus, and nucleoplasm. In certain regions, the nuclear membrane seemed to undergo a form of specialization, characterized by the presence of whorls of the external element of the nuclear envelope positioned in the juxtanuclear cytoplasm. Portions of each whorl of membrane often appeared more electron dense than the rest. In the cytoplasm, endoplasmic reticulum made rough surfaced by the presence of adherent ribosomes was in abundance. The closely packed lamellae and small vesicles of the Golgi apparatus could be seen in most sections. Lysosomes were present in a range of sizes and electron densities, though they were certainly less abundant than in the PMN leucocyte. In many lysosomes a limiting membrane could be

visualized but in others the membranes were so closely adherent to the dense contents that it could not be distinguished. Pinocytic vesicles and digestive vacuoles with single limiting membranes, which tended to be the least dense elements of monocytes from peripheral blood were common. Small round mitochondria with typical profiles were the other organelles usually seen. Free ribosomes, appearing as tiny, dense particles, were found scattered throughout the cytoplasm, even into the pseudopodia and cytoplasmic veils where organelles such as lysosomes or pinocytic vesicles were rarely seen.

The lymphocyte (Figure 2) assumed a more spherical shape, and its surface had fewer irregularities and convolutions, though some short pseudopodia were present. Its large, kidney-shaped nucleus occupied most of the cell volume. Whorls and reduplications of membrane were commonly seen in the juxtanuclear cytoplasm, adjacent to and often continuous with the outermost layer of the nuclear envelope. The amorphous, dense chromatin material within the nucleus occupied both peripheral and central locations. The thin rim of cytoplasm between nuclear membrane and plasma membrane contained numerous free ribosomes, seen as small, discrete, electron dense deposits, but few organelles. The mitochondria were seen more commonly than other organelles. A small Golgi apparatus with parallel lamellae and small vesicles, was observed positioned in relation to the nuclear indentation. Rarely, a centriole like that seen in Figure 2 was encountered. Occasional membrane bound vesicles which appeared similar to the vesicles of the Golgi apparatus, were seen scattered throughout the cytoplasm in most cells.

The eosinophil (Figure 3), like the lymphocyte, was usually spherical in shape and regular in outline, and its surface projections as pseudopodia and cytoplasmic veils were few. The nucleus, though multilobed as seen by light microscopy, rarely was seen to possess more than two lobes in any section. Its nuclear membrane enclosed dense, amorphous chromatin material which was most often peripherally located. Figure 3 demonstrates a finding within the nucleus which was seen rarely in eosinophils but commonly in certain PMN leucocytes. Superimposed on the dense chromatin was a skein of a fine latticework of dense, threadlike material. Whorls of membrane in the juxtanuclear cytoplasm were rarely seen in the eosinophil. The cytoplasm was dominated by large, characteristic membrane bound granules. These granules contained an amorphous, electron dense material subadjacent to the limiting membrane, and a denser core material. This core material, rod shaped in longitudinal section, resembled crystalline protein described by other investigators. Mitochondria, Golgi apparatus, and small vesicles with pale contents and irregular contour were the other organelles commonly encountered. Unlike the monocyte or lymphocyte, the cytoplasm of the eosinophil had no free ribosomes; the small dense deposits in the cytoplasm probably represented free glycogen deposits.

The PMN leucocyte (Figure 4), generally ovoid in shape, usually had an irregular contour secondary to the many broad-based pseudopodia that were present. Cytoplasmic veils, i.e. the long, thin cytoplasmic projections possessing a narrow base, were rare in the normal PMN leucocyte. Sections through the multilobed nucleus frequently crossed

four to six lobes. Perinuclear whorls of membrane, occasionally seen in continuity with the outer layer of the nuclear envelope, were common. In most cells, evidence of nuclear-cytoplasmic interdigitation was seen. Such interdigitations created the impression that islands of cytoplasm were sequestered within the nucleus. With the brief refixation in osmium found requisite for proper thin sectioning, the chromatin material within the nucleus and all membranes in the cytoplasm were usually indistinctly seen. The cytoplasm was packed with granules specific for the PMN leucocyte, containing different densities of amorphous material. Though the limiting membrane of these granules was occasionally seen, it was usually obscure. Subclassification of these granules on the basis of their relative density, size, or other characteristic was not attempted in view of the variable appearance in different fixations. In the normal peripheral blood PMN leucocyte, pinocytic vesicles were uncommon. Ribosome-studded endoplasmic reticulum often appeared to have broad cisternae between the two membranes, a characteristic shared by sections of the nuclear membrane. Elements of the endoplasmic reticulum and Golgi apparatus were not often seen, however, and were usually small when encountered. The ground hyaloplasm of PMN leucocytes, seen as the background density in regions not occupied by discrete deposits or organelles, was denser than that of the other blood cells examined. Glycogen deposits which along with the specific granules are a characteristic feature of the cytoplasm of any normal PMN leucocyte were seen as small, discrete, electron dense particles which stained heavily with lead.

In an effort to visualize the chromatin material, poorly seen in lightly osmicated specimens, phosphotungstic acid (PTA) was sub-

stituted for osmium tetroxide during tissue preparation. PTA is known to bind with the histones of DNA and other basic proteins, though its use in leucocytes has not been reported. Characteristically, it stains no membranes in the cells, as can be seen in the next three plates.

The monocyte (Figure 5) had an irregular border with pseudopodia and cytoplasmic veils, though the plasma membrane was not visualized. The chromatin material within the nucleus was peripherally arranged subadjacent to the lucent nuclear membrane, but interrupted at intervals. At these points, most often located in regions of nuclear membrane concavity, there was little or no dense chromatin material apposed to the nuclear membrane. Also in these areas, cytoplasmic density was often increased and the cistern of the nuclear membrane could be clearly distinguished as a thin linear density between two thin linear lucencies. It was thought likely that these areas represented pores in the nuclear envelope. Because the mitochondrial cristae and limiting membranes were not stained but the matrix was, the mitochondria appeared striped. The many lysosomes were also intensely reactive with PTA and assumed many shapes, from elongate rods to spherical bodies. Centrioles with microtubules were easily demonstrated in many sections, because of their affinity for PTA.

Eosinophil (Figure 6) granules appeared the opposite of those fixed in osmium tetroxide. Their core crystalline material appeared non-opaque, and was surrounded by a densely reactive amorphous material. Eosinophil nuclei demonstrated peripherally distributed chromatin patterns similar to monocytes. In those areas in which chromatin

material was not apposed to the nuclear membrane, a dense material appeared in the juxtanuclear cytoplasm at the margin of suspected nuclear pores. A few matrices of mitochondria and contents of vesicles were reactive, but the rest of the cytoplasm appeared fairly homogenous, with occasional foci of denser ground hyaloplasm.

In contrast to the nuclei of the other types of leucocyte stained with PTA, the PMN leucocyte nucleus displayed a clumped, centrally distributed chromatin pattern (Figure 7). Though a narrow fringe of amorphous density was apposed to the nuclear membrane along its entire circumference, the majority of the dense amorphous nuclear material was clumped centrally. In addition, the density of the relatively lucent nucleoplasm was less than in the other types of leucocytes, and it appeared less granular. There were typically no areas in which the narrow peripheral fringe of chromatin material was interrupted. Although there were areas where the cistern between the layers of the nuclear membrane stained densly, this occurred in places where the dense, central clump of chromatin material touched the nuclear membrane, rather than in places where chromatin density was absent as in the other types of leucocyte.

Within the cytoplasm of the PTA stained PMN leucocyte a great variety of structures could be seen, the identity of which could not be established with certainty. The extremely dense round to oval bodies were probably PMN leucocyte granules (lysosomes). The large round structures with dense amorphous material and those structures with other irregular configurations of density were thought to be digestive vacuoles. In Figure 7, the area of alternating lamellae of

density and lucency with associated reactive contents of tiny vesicles was seen only rarely in other PMN leucocytes and was thought to represent Golgi apparatus. A multitude of smaller organelles of differing densities were observed, which may have represented pinocytic vesicles, phagocytic vacuoles, or perhaps small lysosomes. Many small dense, apparently membrane bound structures, whose length exceeded width by ten times or more, appeared to have no correlate in osmium treated preparations. At least some of these structures may have represented stained cisternae of encoplasmic reticulum.

Of additional interest was the density and granular appearance of the ground hyaloplasm when compared to the other types of leucocyte examined. Focal areas of increased density were located at intervals along the plasma membrane, especially in areas relatively close to the nucleus.

Normal Rabbit Leucocytes

The morphology of normal rabbit leucocytes which were separated from whole blood by dextran sedimentation and fixed with glutaraldehyde and refixed in osmium tetroxide will be presented.

The rabbit monocyte (Figure 8) was quite similar in appearance to the human monocyte, being generally oval in shape and possessing an irregular border. The cell surface was thrown up into many cytoplasmic veils and a few pseudopodia. The nucleus with characteristic central indentation was surrounded by a double-layered nuclear envelope. In relation to this envelope, whorls and reduplications of membrane similar to the types described in human leucocytes were seen. The nuclear contents appeared amorphous and granular, and no chromatin

pattern could be distinguished. The widely separated parallel membranes of the endoplasmic reticulum were in abundance. Large mitochondria, a few dense lysosomes, and many pinocytic vesicles were seen. Free ribosomes were very common.

The rabbit lymphocyte (Figure 9), also similar to its human counterpart, possessed a relatively smooth border with few cytoplasmic veils, large nucleus, and thin rim of cytoplasm containing a few mitochondria, a Golgi apparatus, and many free ribosomes. The re-duplications of membrane in a juxtanuclear position commonly seen in lymphocytes were well resolved in this cell. Such an area was seen under high magnification (Figure 10) to consist of tubular membranes, some of which appeared denser than others. These tubular membranes were intertwined within an expanded area, seemingly outside of the cistern of the nuclear envelope which narrowed adjacent to the special-ization. The neighboring Golgi apparatus (Figure 10) was composed of lamellae of membranes enclosing cisternae, and small vesicles which appeared to have been budded from the ends of the membranes.

The rabbit eosinophil (Figure 11), on the other hand, was quite different from its human counterpart. A dense, osmophilic material occupied the entire volume of the characteristic granules. There seemed to be a substructure of the contents of the granule, as platelike crystalline densities were superimposed on the background density with-in the granules. No real distinction between core and peripheral substances could be made, as was possible in the human eosinophil.

Often known as the heterophil based on its appearance in Wright's stain, the rabbit PMN leucocyte (Figure 12) was also quite different

from its human counterpart. Fixation to a tissue hardness compatible with thin sectioning proved difficult, and the appearance of this cell varied markedly with small changes in fixation time.

The shape of the PMN leucocyte, generally ovoid, was irregular because of the presence of many pseudopodia. Few cytoplasmic veils were observed. The multilobed nucleus was surrounded by a poorly defined nuclear membrane with a large central cistern. The nucleus contained a peripherally arranged dense amorphous chromatin material and less dense finely granular nucleoplasm. In areas where the nucleoplasm was apposed to the inner layer of the nuclear envelope, the cistern between the layers of the nuclear envelope seemed to contain dense material absent in other areas. These areas were thought to represent nuclear pores. The cytoplasm contained two types of membrane bound granules. A larger dense type was more refractory to thin sectioning, and filamentous densities seen within them may have represented uneven section thickness. The population of smaller granules was generally slightly less dense; only a few granules were more lucent than the cytoplasmic ground hyaloplasm. No "target-type" granules with dense core and lucent periphery were seen. Elements of endoplasmic reticulum, mitochondria, and Golgi apparatus were rarely identified. Discrete granular deposits made more dense by staining with lead citrate were scattered throughout the cytoplasm, and were probably glycogen deposits.

Rabbit Lung Macrophage

As noted previously, the mononuclear cells obtained from saline washings of the rabbit bronchoalveolar system have been shown to be

capable of pyrogen release under suitable stimulation. A preparation of non-sensitized lung mononuclear cells was obtained on one occasion with extremely low contamination from peripheral blood. This preparation was examined with the electron microscope to identify the types of mononuclear cells present. On the basis of observations of many sections it can be stated that 90-95% of the cells seen were of the type shown in Figures 13 and 14. On the basis of their fine structural appearance, they could be classified generally as macrophages, and closely resembled the wandering alveolar macrophage found in preparations of intact lung. They were quite different from both monocytes observed in rabbit blood and other nonphagocytic cells which inhabit the rabbit respiratory tract. This large cell contained a considerable amount of endoplasmic reticulum with adherent ribosomes, uniformly dense lysosomes of differing shape, mitochondria, and some digestive vacuoles. Many small membrane bound vesicles were seen throughout the cytoplasm. The surface of the cell was irregular, thrown up into many pseudopodia and cytoplasmic veils. A cross-section of some of the cytoplasmic veils at high magnification (Figure 15) demonstrated a fine, fibrillar mucoid substance coating the plasma membrane and extending into the extracellular substance. Typically, no organelles could be found within the narrow cytoplasmic veils.

Pyrogen Producing Cells

Rabbit peritoneal exudate

Rabbit peritoneal exudates, composed promarily of PMN leucocytes which are actively releasing EP, were harvested four hours after intraperitoneal instillation of sterile glycogen. As previous results have

shown, these exudate cells are "activated" and would have continued to release EP on incubation without further stimulation. Because of the likelihood of cell damage during incubation, the exudates were fixed immediately after washing with saline. However, in spite of efforts to equalize tissue preparation schedules for the exudate leucocytes and for circulating leucocytes obtained by cardiac puncture, the fine structural appearance of the cells was quite different, especially with regard to relative electron density. Thus, it was difficult to know whether differences between control and activated cells could be attributed to cell activation *per se*, or whether these differences represented incidental findings related to the different media in which the cells were originally present.

The exudate PMN leucocyte (Figures 16 and 18) was generally larger in size than the normal blood PMN leucocyte (Figures 17 and 12). The exudate cell had a more irregular border because of the presence of greater numbers of pseudopodia. Its cytoplasm contained many digestive vacuoles within which the exogenous, phagocytosed glycogen used to induce the formation of the exudate could be seen. The control cell, on the other hand, contained no such digestive vacuoles filled with exogenous glycogen. Its cytoplasm contained dense deposits of endogenous glycogen free in the hyaloplasm. These endogenous deposits were distinctly less common in the exudate cell.

For unknown reasons, the PMN leucocyte of the peritoneal exudate was considerably less refractory to thin sectioning than the normal blood PMN leucocyte. As a result, a clear distinction between the two populations of cytoplasmic granule (lysosome) was more easily made.

The relative proportions of the large, dense, and less common type and the smaller, less dense, and more common type were similar in both exudate and control PMN leucocytes. From impressions gained by examination of large numbers of micrographs from exudate and control populations, it seemed that the exudate cell contained more granules of all types, more elements of endoplasmic reticulum, and more frequently encountered elements of the Golgi apparatus (cf. Figures 12, 16-18). No amorphous, finely granular material was found either intra- or extra-cellularly which resembled the substance described by Goodale, Hillman, and Fillmore (70,71).

Etiocholanolone-activated cells

As stated previously, it has not been established which cells in human peripheral blood are activated by etiocholanolone to release EP. Examples of each of the four most common types of cell encountered will be presented. Both control and experimental cell preparations were treated identically except for the addition of etiocholanolone to the experimental incubation medium. Pyrogen testing revealed the supernatant from the control incubation to be non-pyrogenic, and the supernatant from the etiocholanolone incubation to be pyrogenic.

The etiocholanolone-treated lymphocyte (Figure 20) was not different with regard to fine structure from the control lymphocyte (Figure 19) in any consistent way. The incidence of juxtanuclear membrane whorls, cytoplasmic veils, and various organelles appeared to be identical in the two populations.

Observations on the two populations of eosinophil (Figures 21

and 22) demonstrated that the chromatin distribution of the etiocholanolone-treated cell tended to be less condensed than that of the control cell. However, eosinophils were encountered rarely in both preparations, and sampling was therefore insufficient. Otherwise, there was no consistent difference observed; the apparent difference in the fragmentation of the core material of the eosinophil granules seen in the examples presented was not consistent, as reference to micrographs of other control eosinophils (Figure 3) substantiated.

The monocyte, which preliminary observations have implicated in pyrogen release during etiocholanolone stimulation (33) was seen frequently in most sections of both control and etio-treated preparations. The monocyte incubated with etiocholanolone (Figure 24) had a similar oval shape and similar numbers of plasma membrane irregularities in the form of pseudopodia and cytoplasmic veils to the control monocyte (Figure 23). The nucleus of the etio-treated cell possessed a less condensed chromatin distribution than the nucleus of the control cell. Many more perinuclear whorls of membrane of the type described earlier were seen in etio-treated monocytes, and where present occupied a larger segment of the membrane and contained denser, more closely packed membrane elements. In the cytoplasm, the mitochondria of the etio-treated monocyte were larger than those of the control cell, and the mitochondrial cristae were packed less closely. Elements of the rough surfaced endoplasmic reticulum were more commonly encountered in the monocyte incubated with etiocholanolone, and were often seen in stacks of parallel double membranes, an infrequent finding in control cells. The prevalence of lamellae

and vesicles of the Golgi apparatus in sections from etio-treated cells seemed greater. No consistent difference between the two populations was found in the number or type of lysosomes or small membrane bound vesicles or the number of free ribosomes present in the cytoplasm. The ground hyaloplasm of the etio-treated monocyte was, however, consistently less dense than that of the control cell.

The PMN leucocyte treated with etiocholanolone (Figures 25 and 27) was strikingly different from the control cell (Figure 26). One of the most consistently observed differences in these cells of similar size was the appearance of multiple cytoplasmic veils at the surface of the etiocholanolone-treated cell. Seen best when sectioned tangential to the cell surface (Figure 25), these cytoplasmic veils were rare in control PMN leucocytes, whose surface irregularities were usually caused by pseudopodia.

These veils arose from a narrow base of cytoplasm and extended from the surface of the cell into the extracellular substance as a thin projection of uniform width which contained distinguishable cytoplasmic organelles only rarely. On the surface of the veils, a fine mucoid extracellular space substance (M.E.S.S.) was routinely seen. The M.E.S.S., however, bore no resemblance to the finely granular material outside leucocytes reported by Goodale, Hillman, and Fillmore (70,71). In many areas, two veils arising from different loci along the cell surface appeared to be in the process of fusion, enclosing a volume of extracellular fluid and debris. In other areas, such enclosed material appeared fully enclosed by membrane and either partially or totally incorporated into the cytoplasmic contents as a vacuole. It was probably the presence of the many vacuoles that

accounted for the larger size of the PMN leucocytes incubated with etiocholanolone.

The nuclear chromatin was less condensed in the PMN leucocytes treated with etiocholanolone, and juxtanuclear whorls of membrane were more commonly seen than in the control cell. Within the cytoplasm, deposits of glycogen which stained densely with lead citrate were smaller and significantly less prevalent in the etio-treated PMN leucocyte. Elements of endoplasmic reticulum, with wide cisternae characteristic of the PMN leucocyte, were commonly encountered in etio-treated cells, but seen rarely in control cells. In general, there were fewer granules (lysosomes) in the etio-treated PMN; although not all cells demonstrated this finding, there were other etio-treated cells which contained significantly fewer lysosomes than any of the control PMN leucocytes seen. No consistent differences in the prevalence of elements of the Golgi apparatus or the numbers of mitochondria were found. In general, the ground hyaloplasm of the etio-treated PMN leucocyte was less dense than that of the control cell.

Human Leucocytes Incubated with Staphylococci

Lymphocytes incubated with staphylococci showed no consistent difference from those which were not; few eosinophils were observed.

The monocytes incubated with staphylococci in the ratio 10/leucocyte did not appear to phagocytose many organisms, for digestive vacuoles containing staphylococci were encountered in only half of the sections. The bacteria were apparently phagocytosed more rapidly by the more common PMN leucocytes. However, a few differences between control (Figure 28) and staph-treated (Figure 29) monocytes were

observed. The size, shape, and number of pseudopodia were similar in both groups, and cytoplasmic veils were rare. The chromatin material of the staph-treated monocyte was less condensed than that of the control, although its peripheral distribution was similar. No difference in the prevalence of reduplications of the nuclear membrane was observed. Within the cytoplasm, the number of lysosomes seemed greater in the control cell. No consistent difference in the number or size of mitochondria, prevalence of elements of the endoplasmic reticulum or Golgi apparatus, or numbers of small membrane bound vesicles was recognized. The density of the ground hyaloplasm and numbers of free ribosomes were also similar.

The PMN leucocyte incubated with staphylococci and fixed in glutaraldehyde and osmium, as mentioned previously, abounded with digestive vacuoles containing the phagocytosed bacteria (Figures 30 and 32). The surface of the PMN leucocyte incubated with staphylococci was more irregular than the surface of the control cell (Figure 31) because of the presence of many pseudopodia. Cytoplasmic veils, so common in PMN leucocytes incubated with etiocholanolone, were rare in both staph-treated and control PMN leucocytes. The nuclei and nuclear membranes did not appear consistently different in the two populations. Fewer deposits of endogenous glycogen were present in the staph-treated cells. Elements of endoplasmic reticulum, usually with wide cisternae, and of Golgi apparatus were more commonly encountered in the staph-treated PMN leucocyte. As was the case in etio-treated PMN leucocytes, the staph-treated cells in general contained fewer lysosomes. Certain cells, like that seen in

Figure 30, clearly contained fewer lysosomes than any control cell, but in others (Figure 32), the pattern was not evident. Of interest is the observation that PMN leucocytes incubated with staphylococci did not stain as heavily in lead citrate as controls, and overstaining with lead citrate did not occur as frequently during equivalent treatments with stain.

The use of phophotungstic acid as a stain for PMN leucocytes in preparations incubated with and without staphylococci proved a fruitful approach to the examination of fine structural differences between the two populations. Unlike the osmium preparations which tended to show wide variations within each population in electron density of various cellular components, the PTA treated cells were remarkably uniform in appearance. Thus, even small differences between staph-treated and control populations could be identified with greater certainly. PMN leucocytes incubated with staphylococci and stained with PTA (Figures 33 and 35) appeared quite different from the control (Figure 34).

In the control cell, as in the normal human PMN leucocyte described earlier, the dense nuclear chromatin was heavily clumped. The majority of the material occupied a central position within the nucleus and was apposed to the nuclear membrane in only a fraction of its circumference. A small proportion of the dense chromatin existed as an uninterrupted fringe along the entire remaining circumference of the nuclear membrane not apposed to the dense, central clump. The nucleoplasm was relatively electron-lucent, and lacked the finely granular texture of other leucocytes stained with PTA. In no areas did the

nucleoplasm appear to touch the nuclear membrane without interposed chromatin density.

In the staph-treated cell, on the other hand, the chromatin material was not as clumped, and its distribution was largely peripheral, being apposed to the nuclear membrane throughout most of its circumference. The nucleoplasm was finely granular in appearance, and some clumps of granularity nearby the peripheral chromatin mass appeared to arise from the chromatin. In addition, several areas in which the chromatin was not apposed to the nuclear membrane, and in which the nucleoplasm was in contact with the membrane were seen in the staph-treated PMN leucocytes. In some of these areas, an increased cytoplasmic density was seen, and the cistern of the nuclear membrane appeared to possess an electron density absent from other regions. These areas of absent chromatin most often occurred in concavities of the nuclear membrane, and were thought to represent nuclear pores, which were rarely seen in control cells.

Centrioles and microtubules, which stained heavily with PTA, were seen far more frequently in the staph-treated cells. Two such centrioles were found in nearly perfect cross-section (Figure 33) and longitudinal-section (Figure 39). The long, thin microtubules radiated from the centriole, and could be seen in areas of the cell at great distance from their presumed origin.

The third major difference observed between control PMN leucocytes and PMN leucocytes incubated with staphylococci concerned the density of the ground substance. The control cell typically possessed a ground substance that was quite dense with respect to that of staph-

treated PMN leucocytes and to that of other leucocytes. In addition, the density of the ground substance was not nearly as homogenous in the control cell as in the staph-treated cell. Areas of slightly increased or decreased density from that of other areas of the ground substance were common in control PMN leucocytes, but less so in cell incubated with staphylococci.

The contents of lysosomes, many of which reacted heavily with PTA, were in general seen more commonly in control cells, but because identification of lysosomes was easier in osmium-fixed material, this observation was not explored in depth in PTA-stained material.

The three major differences in PTA morphology between the two populations (nuclear chromatin distribution, prevalence of centrioles and microtubules, and density of ground hyaloplasm) were remarkably constant. Over 95% of the control PMN leucocytes demonstrated the patterns described as characteristic of that population; similarly 95% of the staph-treated cells showed characteristic patterns. The remaining 5% of each group, however, demonstrated patterns in reverse of expectation. Thus, a few control cells were seen with peripheral chromatin distributions, many microtubules, and a relatively lucent ground substance, the pattern characteristic of staph-treated cells. A few staph-treated cells appeared like control cells (e.g.Figure 37). Still others appeared to be intermediate in morphology, showing characteristics of both populations (Figure 36).

Other differences between the two populations were observed in PTA stained material, but because these changes involved organelles bound by membrane, they were more closely delineated in osmium treated

preparations. No consistent difference in morphology, number, or type of PMN leucocyte granules was seen, but a minor difference would not be evident in thin sections.

Leucocytes Incubated with Staphylococci and Actinomycin-D

The effect of 5 micrograms/ml. actinomycin-D incubated for 4 hours with normal leucocytes which were then fixed in glutaraldehyde and postfixed in osmium tetroxide, was investigated. The number of viable organisms was similar in aliquots from control and actinomycin-treated incubations.

All four major varieties of leucocyte (Figures 40-43) often contained a strange, threadlike deposit within their nuclei. Seen most often in the PMN leucocyte, the deposit appeared to consist of many small rings of dense, fibrous material connected by fine linear strands of the same material. Neither the size of the rings nor length of the strand was constant, and the overall configuration of the deposit was extremely variable. Because the deposit was seen so frequently in leucocytes treated with actinomycin-D and so rarely in those which were not, it was thought less likely to represent an artefact of tissue preparation.

In PMN leucocytes treated with actinomycin-D (Figure 43), there seemed to be a decrease in the number of lysosomes and an increase in the amount of endogenous glycogen when compared with normal, control PMN leucocytes. Otherwise, there were no consistent differences between control and actinomycin-D treated monocytes, lymphocytes, eosinophils, or PMN leucocytes.

Leucocytes incubated with and without staphylococci for 30

minutes were exposed to actinomycin-D for the remainder of the 4½ hour incubation. Supernatants from both preparations were non-pyrogenic when tested in rabbits after the incubation was concluded.

The monocyte having exposure to staphylococci prior to addition of actinomycin-D was compared to the monocyte exposed to actinomycin-D alone (actinomycin control) in glutaraldehyde and osmium fixation (Figures 44 and 45). The chromatin material of the staph-actinomycin-treated cell was less dense, although its distribution appeared similar to that of the actinomycin control cell. Elements of endoplasmic reticulum and Golgi apparatus were seen more commonly in the staph-actinomycin-treated monocyte than in the actinomycin control cell. The plasma membrane was more irregular because of the increased number of pseudopodia and cytoplasmic veils, and there were more digestive vacuoles in the staph-actinomycin-treated cell. No difference in number or size of mitochondria or lysosomes, or in the density of the ground substance were observed.

The PMN leucocyte exposed to staphylococci and actinomycin-D (Figures 46 and 48) was compared to the PMN leucocyte treated only with actinomycin-D (actinomycin control) (Figure 47). The differences between these cells represented morphological changes induced by staphylococci in actinomycin-treated cells. Since both staph-actinomycin treated and actinomycin control preparations were non-pyrogenic, any differences between the two cell types would not be intimately related to EP release.

The PMN leucocyte exposed to staphylococci and actinomycin-D was larger than the actinomycin control leucocyte, presumably because

of the presence of many phagocytic vacuoles containing staphylococci. The surface of the staph-actinomycin treated cell was more irregular because of the presence of more pseudopodia than were present in the actinomycin control cell. Elements of endoplasmic reticulum and Golgi apparatus were more frequently encountered in the staph-actinomycin-treated cell, but more endogenous glycogen was found in the actinomycin controls. Affinity for lead citrate was more pronounced in the actinomycin control PMN leucocytes, and the tendency to overstain was greater than in the staph-actinomycin treated cells. Fewer lysosomes were ordinarily present in staph-actinomycin treated cells, and in some sections fewer than 5 granules were observed. There appeared to be no difference in the number of mitochondria in the two populations. Of some interest is the observation that the thread-like deposits described in actinomycin treated cells and found frequently in the actinomycin control PMN leucocytes were virtually never seen in staph-actinomycin treated cells.

It may be recalled that the differences between PMN leucocytes treated with staphylococci and PMN leucocyte controls were nearly identical to the differences just described between staph-actinomycin treated and actinomycin control cells. Although the addition of actinomycin-D to staph-treated PMN leucocytes effectively blocked EP release, none of the fine structural changes induced by incubation with staphylococci were blocked. Stated differently, this finding implied that in PMN leucocytes there were no observed morphologic changes intimately related to EP release.

The comparison of staph-treated cells (releasing EP) with staph-

actinomycin treated cells (not releasing EP) confirmed this observation. No differences could be seen which could not be attributed to an effect of actinomycin-D alone on normal cells. The staph-actinomycin treated cell (Figure 50) is not different from the staph-treated cell (Figure 49) in any consistent way except for a larger number of granules in the staph-treated cell. This difference, observed as an effect of actinomycin-D on normal cells, was therefore an expected finding. Higher magnification views of the cell surface in three populations (staph-treated, actinomycin control, and staph-actinomycin treated PMN leucocytes) were also studied (Figures 51-53). They confirmed the view that actinomycin-D blockade of EP release did not modify any of the morphologic changes observed after incubation with staphylococci.

Comparisons among the different populations of PMN leucocyte stained with PTA resulted in the same conclusions. The PMN leucocyte exposed to staphylococci and actinomycin-D, fixed in glutaraldehyde, andstained with PTA (Figures 54 and 56) was compared to the PMN leucocyte treated with actinomycin-D alone, and fixed and stained identically (Figure 55). The actinomycin control cell exhibited chromatin material distributed in one large central nuclear clump, with a narrow fringe of chromatin apposed to the circumference of the nuclear membrane, that was characteristic of normal cells described earlier. The paucity of microtubules and the relatively great density of the ground substance, also a characteristic of normal cells, was observed in actinomycin control cells.

On the other hand, the staph-actinomycin treated PMN leucocytes

demonstrated a peripheral chromatin distribution and finely granular nucleoplasm. Areas of nuclear membrane which were free of chromatin apposition possessed the same characteristics of similar areas seen in control cells. Microtubules and centrioles were commonly seen in staph-actinomycin treated cells, and the ground substance was more lucent than that of actinomycin controls.

In short, the appearance of PTA-stained actinomycin control cells resembled that of control cells (described earlier), and the appearance of staph-actinomycin treated cells was like that of staph-treated cells (also described earlier). Since the non-pyrogenic staph-actinomycin treated PMN leucocyte resembled the pyrogenic staph-treated cell, it appeared that the morphologic changes induced by staphylococci were not modified by the addition of actinomycin, even though EP release was completely blocked by the presence of actinomycin.

The comparison of staph-treated PMN leucocytes stained with PTA (Figure 57) and staph-actinomycin treated PMN leucocytes similarly stained (Figure 58) was also performed. No difference was noted except for the lower number of lysosomes in the staph-actinomycin treated cell, an effect noted in normal cells treated with actinomycin.

Thus, in PTA as well as in osmium treated material, actinomycin blockade of EP release did not modify any of the morphologic changes induced by incubation with staphylococci. Higher magnification views of the surface membrane confirmed this view (Figures 59-62). Both control and actinomycin control exhibited a dense ground substance, especially near the plasma membrane, but this density was not observed in either staph-treated or staph-actinomycin treated PMN leucocytes.

DISCUSSION

The investigation of fine structural alterations accompanying the event of EP release in various leucocytes was the purpose of this study. Early results indicated that many morphologic changes occurred in leucocytes when they were "activated" to release EP. Therefore, experiments utilizing metabolic blockade of EP release by actinomycin-D were performed to identify which of the morphologic changes were closely related to EP release per se and which were manifestations of a more generalized activation state involving other leucocyte functions not inhibited by actinomycin-D. In the process of the investigation, several previously unreported details of normal leucocyte ultrastructure were found; these will be discussed following a short comment on methodology.

Leucocyte fixation

Considerable difficulty was encountered in the proper fixation of blood leucocytes in glutaraldehyde and osmium tetroxide. Although schedules were developed which produced acceptable morphological preservation, a lack of uniformity in the relative electron densities of cell constituents within each and among different preparations was found. This variability, most pronounced in the PMN leucocyte, was secondary to presumed differences in fixation.

Since the work on this study was completed, the author learned of an as yet unpublished method developed by Hirsch and coworkers (85) which shows great promise in attaining reproducibility of fixation. Leucocytes in suspension were exposed to an osmium tetroxide and glutaraldehyde mixture, followed by treatment with uranyl acetate, dehydration, and epoxy resin embedment. Hirsch had also encountered fixation

problems with all presently published preparation schedules for the leucocytes.

No such variability was encountered in preparations fixed in glutaraldehyde and stained with alcoholic PTA. Because this method was first tried late in the progress of this study, it was not possible to apply this fruitful technique to other than human leucocytes and the activation system involving staphylococci.

Normal Leucocytes

The present observations made on the normal human peripheral blood leucocytes fixed in glutaraldehyde and osmium tetroxide were similar to those reported by Anderson (2). However, an additional previously undescribed feature was the appearance of whorls and reduplications of membrane adjacent to the nucleus in most normal human leucocytes examined. Since such specializations were observed in all methods of tissue preparation, they were not thought to be artefacts of the method used. Similar structures were also seen in normal rabbit leucocytes. Well-resolved whorls and reduplications were composed of tubular membrane elements which appeared to lie external to the nuclear envelope. Because communication with the nuclear envelope and its cistern may have been present but out of the plane of section, it could not be determined whether the structure was actually a specialization of the outer layer of the nuclear envelope or a contiguous specialization of cytoplasmic membrane elements. It was intriguing to speculate that these membrane specializations play a role in nuclear-cytoplasmic interaction.

As previously mentioned, the use of 1% PTA in absolute ethanol

as a stain for glutaraldehyde-fixed leucocytes proved a worthwhile technique that provided new information. Although use of this technique has not been reported in human leucocytes, the structures within leucocytes which reacted were probably basic proteins, such as histones, as indicated by previous work (146). The structures which reacted were the lysosomes, the centrioles and microtubules, and the nuclear chromatin material. A surprising amount of density was imparted to the hyaloplasm in leucocytes, particularly in the PMN leucocyte.

The specificity of PTA reaction with the histones of the nucleus has been found to be similar to the histone affinity of fast green in light microscopy (146). In view of this knowledge, the dense "chromatin material" as defined by PTA staining was probably a more reliable indicator of interphase chromosomes than the "chromatin material" defined as nuclear density in osmium tetroxide fixed preparations. The brief osmium tetroxide treatment administered to cells in this study often produced no recognizable chromatin distribution, whereas areas of nuclear density were always prominent in PTA treated material.

The distribution of chromatin material in the normal human eosinophil and monocyte stained with PTA was markedly different from that in the normal PMN leucocyte. In eosinophils and monocytes, the peripherally arranged chromatin masses were discontinuous in their apposition to the nuclear membrane. In those areas where nucleoplasm was in contact with the nuclear membrane without interposed chromatin, the usually lucent cistern of the nuclear membrane was often dense. The contiguous cytoplasmic hyaloplasm was also increased in density. In

well-resolved areas, the density of hyaloplasm and nucleoplasm appeared to be continuous. Such a structure was thought to be compatible with that of an opening, or pore, in the nuclear envelope. Whether such nuclear pores have a function is uncertain, but it seems reasonable that they represent sites of nuclear-cytoplasmic interaction.

Whatever the function of these areas, they were not seen in the normal PMN leucocyte stained with PTA, in which the majority of the chromatin mass was clumped centrally, and a small fringe of chromatin density was apposed to the nuclear membrane throughout its entire circumference. The nucleoplasm was relatively lucent, and lacked the finely granular appearance of nucleoplasm in monocytes and eosinophils. Since the fine granules of nucleoplasm in other cells appeared to arise from the chromatin mass, it may be that the lack of granularity in the normal PMN leucocyte nucleoplasm reflected a structural condensation and clumping of the histone-containing nucleoprotein.

The fine structure of rabbit blood leucocytes has not been reported, though several studies of the rabbit peritoneal exudate PMN leucocyte have appeared. Rabbit and human blood monocytes and lymphocytes were similar, but the eosinophil and the PMN leucocyte from these two sources were somewhat different. The entire content of the rabbit eosinophil granules was dense, and still denser material in a crystalline array was superimposed on the background density. In the human eosinophil granules a clear distinction between dense, crystalline core material and lucent peripheral material could be made, but this relationship did not apply to rabbit eosinophil granules.

Within the rabbit blood PMN leucocyte, there were clearly two

types of granules similar to those described by Bainton and Farquhar in rabbit exudate PMN leucocytes (11). The larger, dense type was more refractory to thin sectioning than the smaller , less dense type. No "target" granules similar to those previously reported (86,87) were seen. Aside from the different morphology of the granules, the rabbit and human PMN leucocyte appeared quite similar.

Mononuclear cells from washings of the bronchalveolar system of rabbit lung have been found to be capable of pyrogen release (6). A preparation of such cells was obtained with identical methods and processed for electron microscopy. Extremely low contamination from pulmonary blood was encountered. Under the electron microscope, at least 95% of cells appeared to be macrophages similar to those described by Karrer (97), and did not resemble monocytes found in rabbit blood. Of course, these macrophages could have arisen by modulation or differentiation of blood monocytes (16). However, on the basis of the fine structural appearance of the cells without regard to their origin, it was felt that the cell in lung washings responsible for pyrogen release was the alveolar macrophage.

Leucocytes activated to release EP

In order to correlate EP release with changes in the fine structure of cells, an activating agent was added to one group of cells, which was compared with a control group of cells obtained from the same source and handled identically. Fine structural differences between the two populations could thus be interpreted as the effect of the activating agent alone.

Activating systems for study were chosen using criteria of cell

viability during incubation and diversity of presumed pathogenesis of activation. Those studied were rabbit peritoneal exudate, human blood incubated with etiocholanolone, human blood incubated with staphylococci, and BCG-sensitized rabbit lung cells incubated with old tuberculin. Thus, among the activation systems studied, cells of at least three types were involved in in vivo and in vitro activations caused by particulate, soluble, and immunological stimuli.

The etiocholanolone activation system was the only one in which experiments were performed only once. In all other systems, results from repeated experiments paralleled those of the original experiment. Pyrogen testing of supernatants from incubation flasks was performed at least once from each activation system: supernatants from controls were non-pyrogenic, whereas supernatants from activated populations produced significant fever. Because of these consistent results with bioassay it was possible to ascribe the morphologic differences between the two preparations to events associated with activation of the cell.

Differences encountered between control and activated cells involving relative numbers of certain cytoplasmic organelles were theoretically subject to statistical evaluation. However, the variability of electron density (secondary to variability of osmium tetroxide fixation) might have introduced a hidden bias. This possible bias, plus a relatively small sample size in instances of certain cells, would have made statistical conclusions difficult to interpret. Simple observations were therefore presented as impressions without statistical confirmation.

It was difficult to obtain equivalent fixation in cells from

peritoneal exudate and from whole blood of rabbits. Although disappointing, this finding was not surprising because of the difference of the media from which they were obtained.

Evidence of an active cell surface as exemplified by the presence of cytoplasmic veils was seen commonly in etiocholanolone-treated PMN leucocytes. These cytoplasmic veils were seen rarely in normal PMN leucocytes and in PMN leucocytes from other activation systems. Their presence has been associated with a high rate of pinocytosis in other tissues (52). It was intriguing to speculate that this pyrogenic hormone had induced surface activity in these PMN leucocytes. No such effect was seen in either monocytes, lymphocytes, or eosinophils incubated with etiocholanolone.

Etiocholanolone was unique among the activating agents studied by virtue of its solubility; phagocytosis was presumably not necessary to initiate activation. Yet morphological changes were observed which were similar to those seen in cells activated by phagocytosis of staphylococci. The morphologic changes of activation were thus found to occur in the absence of phagocytosis. A similar finding has been reported recently in the biochemical literature. Graham et al observed that metabolic changes similar to those seen following phagocytosis occurred after incubation with certain soluble agents (72).

For unknown reasons, neither control or tuberculin-activated lung macrophages survived the tissue proparation procedures with acceptable morphology even though there was no evidence of cell lysis before fixation. Fixation procedures followed were the same as for the unincubated lung macrophage (e.g. Figures 13-15) which remained

intact, so it must be assumed that the incubation somehow rendered the cells more fragile.

Fine structural differences between control and activated cells were numerous in all three activation systems studied. However, in both etiocholanolone and staphylococcal activated systems, there was no difference between control lymphocytes and those incubated with the activating agent. Present data suggest that the lymphocyte is not capable of EP release (5). Too few eosinophils were encountered for adequate comparison, but a suggestion of a decreased chromatin density in the etiocholanolone-treated cell was seen.

Monocytes, implicated in pyrogen release when incubated with etiocholanolone (33,172), may be a source of EP in preparations incubated with staphylococci as well (31). Monocytes incubated with both of these activating agents showed a less condensed chromatin pattern, though the peripheral distribution of chromatin was similar to controls. Only in the etiocholanolone-treated cell, however, were juxtanuclear whorls and reduplications of membrane, described in detail earlier, more common than in controls. Mitochondria were larger, elements of the endoplasmic reticulum were more commonly seen, and the hyaloplasm was less dense in the etiocholanolone-treated monocyte. These differences were not observed in monocytes from either control or activated populations of the staphylococcal system. Whether the absence of these differences in the staphylococcal system reflected a non-participation of the monocyte in EP release in this system could not be determined at present. However, little evidence of phagocytosis of staphylococci was seen in the monocytes studied. Monocytes which had phagocytosed

more staphylococci might have shown a greater degree of morphologic change.

PMN leucocytes from all activation systems studied demonstrated certain consistent differences from their respective control populations. Activated cells were in general larger, probably as a result of the presence of many phagocytic, pinocytic, and digestive vacuoles, seen less commonly in controls. Stores of endogenous glycogen as defined by discrete angular deposits staining heavily with lead citrate were significantly decreased in activated PMN leucocytes. This finding correlates with biochemical data obtained from phagocytic cells (95). Cytoplasmic organelles which play a role in the synthetic activity of cells, endoplasmic reticulum and Golgi apparatus, were in general encountered more frequently in activated cells from all three activation systems. In human PMN leucocytes incubated with etiocholanolone or staphylococci, there were fewer granules (lysosomes) than in controls; the opposite was the case in rabbit exudate cells compared with control rabbit blood PMN leucocytes. Hirsch has shown by cinemicrophotography the "degranulation" of phagocytosing PMN leucocytes (84).

The three major differences observed between cells activated with staphylococci and control PMN leucocytes in PTA stained preparations concerned nuclear chromatin distribution, prevalence of microtubules, and density of ground hyaloplasm. These differences were so constant that in 95% of cells examined it was possible to determine purely on the basis of their fine structural appearance which cells were from control preparations and which were from activated preparations. That is, 95% of the control cells exhibited morphologies

which were different with regard to all three major characteristics from 95% of the activated cells. The remaining 5% of cells possessed morphologies contrary to expectation. Rarely encountered cells, which appeared to be intermediate in morphology between typical control and typical activated cells were seen.

The activated cell, in contrast with the control, demonstrated a less condensed chromatin substance suggestive of chromatin activity and fine structural evidence of nuclear cytoplasmic interaction through the pores in the nuclear envelope.

The prevalence of microtubules and centrioles encountered in the activated cells, and the relative paucity in control cells was striking. Several theories of the function of microtubules have been proposed recently. They have been implicated in the maintenance of an asymmetrical cell shape (66,148), in cell motility and motility of portions of cytoplasm (40,104), in the maintenance of an irregular nuclear shape (145), and in channeling and transport of cell material (145). Each of the theories has considerable indirect evidence to support it in the literature of microtubular fine structure (14,15,40, 63,66,82,104,105,130,142,147,148,158,160). Thus, the microtubules may have played a role in phagocytosis through changes in the cell shape, in nuclear-cytoplasmic interaction through changes in nuclear morphology, and in motility and transport in the activated cell with an increased metabolic rate.

The density of the ground hyaloplasm was significantly decreased in activated cells. Since PTA is known to react with basic protein moieties, it may be that activated cells either contain less basic

protein in the hyaloplasm or that this protein is less condensed or structurally different. Thus, the basic protein may have been released from the activated cell into the extracellular space. It is known that many enzymes are released from activated cells (113). On the other hand, the hyaloplasm may have undergone a change in structure accompanying a gel-sol transformation of the substance, known to occur in metabolically active cells.

Blockade of activation by actinomycin-D

It was evident from examination of micrographs of cells from control and activated populations that many changes had occurred when normal cells were incubated with activating agents. Since it was questionable that all of the changes were intimately concerned with the production and release of EP, an attempt was made to achieve an artificial blockade of EP release without also blocking the process of phyagocytosis or the metabolic changes associated with phagocytosis. Actinomycin-D added one half hour after the initiation of incubation of staphylococci with leucocyte suspensions, blocked the release of EP without inhibiting phagocytosis or the increase in the HMP shunt pathway.

Presumably, actinomycin-D exerts blockade of EP release through its well-known effect of blockade of RNA synthesis. Fine structural studies on cells other than leucocytes have shown few, if any, morphological correlates of the profound metabolic inhibition produced by actinomycin-D. In normal human blood leucocytes exposed to actinomycin-D, only two minor changes were observed. There were distinctly fewer lysosomes in cells treated with actinomycin-D. This may have represented a blockade in synthesis of new lysosomes or a cellular

response of "degranulation". Also, within the nucleus of all types of leucocytes fixed with glutaraldehyde and osmium tetroxide skeins of threadlike density were often encountered. Since these densities were not seen in cells stained with PTA, they probably did not represent histones of chromatin; they may have been condensations of nuclear ribonuclear protein or of nucleoplasm.

When actinomycin-D was added after the initiation of incubation of staphylococci and leucocytes, EP release was blocked but none of the fine structural changes seen as a result of incubation with the activator were blocked in either osmium fixed or PTA stained PMN leucocytes. The only difference between cells treated with staphylococci and those treated with staphylococci and actinomycin-D was a decrease in the number of lysosomes in the blockaded population. This effect was certainly due to an effect of actinomycin alone, and was not a result of EP blockade, since normal cells treated with actinomycin had fewer lysosomes than controls. Thus, the event of EP release was separable by artificial metabolic blockade from <u>all</u> the morphologic changes seen after exposure to the activating agent.

The activating agents studied induced striking fine structural changes in PMN leucocytes, but these changes were separable from the event of EP release. However, it might be argued that EP was released in quantities too small to detect by bioassay in cells incubated with activating agent and blocking agent. If so, one might expect to find similar, but less pronounced changes in the blockaded population compared with the unblocked population. In fact, the changes were identical in degree, so it was felt more likely that the changes seen after incubation with the activating agent were probably not intimately related

to EP release.

The morphologic changes induced by the activating agents were indicative of increased levels of synthetic activity, of nuclear cytoplasmic interaction, of motility, and of phagocytosis. These observations led to the hypothesis that each activator of pyrogen release induced a "general activation state" in PMN leucocytes, which involved a number of morphologic changes necessary to initiate or accelerate phagocytosis, increase the rate of energy utilization, adapt the cell to adverse extravascular environments, and release a complement of humoral factors, among which is EP. On the basis of this hypothesis, the event of EP release was thought to be a small but significant part of the leucocyte functions induced by an activating agent.

Although many biochemical changes occur early after exposure to the activator, EP release does not begin until after a lag phase of 30-60 minutes. Since actinomycin-D was added after one half hour, it may have blocked only those leucocyte functions induced by activation which occur after a lag phase. On the other hand, EP production and release may have been induced via RNA synthesis, while the morphologic changes did not require the participation of newly formed RNA, and hence were not blocked by actinomycin-D. Among the morphologic changes induced by the activating agent was an increased prevalence of elements of the endoplasmic reticulum, which is usually associated with an increase in protein synthesis. However, actinomycin-D blocks new protein synthesis through inhibition of new RNA synthesis. The morphological changes suggesting increased protein synthesis thus apparently occurred

without requiring new RNA.

Relationship of Structure and Function in EP Release

Because EP has been characterized as a basic protein and PTA reacts heavily with basic proteins, EP might be expected to be electron dense in PTA stained preparations. However, EP also has an estimated molecular weight of 13,000 (101), so it was unlikely that single molecules or small aggregations of EP would be visible at the limits of resolution in the electron microscope. Large aggregations, however, would presumably be seen in PTA stain. In all preparations either osmium fixed or PTA stained comparing cells releasing EP with those not releasing EP, no material was seen either intracellularly or extracellularly which was suggestive of EP. Therefore, either EP did not exist in large aggregates or it did not stain because of the unavailability of the basic protein groups for reaction with PTA.

One of the theories of EP release postulates an intracellular precursor from which EP is cleaved following cell activation. Because of the small lipid component found in EP, it has been proposed (133) that the precursor molecule of EP exists as a part of the plasma membrane or other membrane element in leucocytes. No definitive loss of density of any membrane structure was observed following activation in PTA stained leucocytes, as might be expected after the release of the basic protein EP. It may have been that the precursor molecule also reacts heavily with PTA, and the loss of a small proportion of that density after release of EP could not be detected. Evidence exists that EP is a very potent molecule; microgram amounts of pyrogenic in vivo (101). On the other hand, the basic groups of EP may

have been unavailable for reaction with PTA in the alcoholic solution used. It is known that a greater number of cellular structures, all of which presumably contain basic protein, react with aqueous PTA (146).

Another theory of EP release postulates the intracellular existence of EP within lysosomes. Following activation, the contents of the lysosomes containing EP are either released extracellularly or diffuse there following intracellular release. Evidence against this theory was found: few examples of lysosomes which appeared to be discharging their contents were found, in either control or activated leucocytes.

EP may be synthesized de novo after activation in leucocytes. Because EP was never visualized, no direct evidence was found to support this theory. However, the increased prevalence of endoplasmic reticulum and Golgi apparatus following activation was suggestive of increased synthesis of protein. Thus, either EP or an enzyme to cleave EP from a precursor may be synthesized after activation. Actinomycin blockade of new RNA synthesis did not block the morphological changes suggestive of protein synthesis, however, and this may represent an uncoupling of biochemical and fine structural events.

Finally, EP may exist in an inactive and an active form. The normal sequence of events following activation may include synthesis of an enzyme to convert EP to its active form, and the subsequent release of the active EP. However, when new RNA synthesis is blocked by actinomycin-D, only the inactive form of EP is released. The decrease in density of ground hyaloplasm found after incubation of PMN leucocytes and staphylococci with or without actinomycin-D may

then be the result of EP release. According to this hypothesis, the presence of actinomycin merely prevents the conversion of the EP to an active form, but does not inhibit the release of PTA-reactive but pyrogenically inactive material from the ground hyaloplasm.

Specifically, no material resembling the amorphous granular substance identified as EP by Goodale, Hillman, and Fillmore (70,71) was seen in any control or activated preparation either intracellularly or extracellularly. The substance reported by Goodale et al was seen in osmium fixed tissue outside human leucocytes activated with endotoxin, outside and (rarely) inside leucocytes from patients with fever, and in "partially purified" EP dried on a grid. No explanation is readily apparent to explain the absence of this substance in the activation systems reported here. In two micrographs from the papers of Goodale, et al, however, sections of cells in the process of autolysis were observed, and the nonspecific granular material they report could have represented cellular debris from other dead cells. In the activation systems studied by Goodale, et al, it is possible that control preparations did not contain such material because they were not subjected to an injurious activator. On the other hand, since the activation systems studied here were not comparable to those of Goodale, et al, the granular material may represent a cellular product other than EP released by certain activators.

Many changes including an increase in cell size, a decrease in stores of endogenous glycogen, an increase in the prevalence of elements of endoplasmic reticulum and Golgi apparatus, a decrease in

the number of lysosomes, an increase in evidence of nuclear-cytoplasmic interaction, an increase in the prevalence of microtubules, and a decreased density of hyaloplasm were encountered in PMN leucocytes following activation. Since none of these changes were blocked during blockade of EP release by actinomycin-D, and since EP itself was not visualized, the definitive identification of an intracellular location of EP and definition of changes associated with EP production and release could not be made at this time. A possible avenue of approach for further study would be through biochemical isolation and purification of EP. This pure EP could then be used to induce anti-EP antibodies in other animals. By administering such antibodies labeled with ferritin to preparations of control and activated cell, the presence of dense antigen-antibody complexes intracellularly could achieve localization of EP and definition of changes associated with its release.

SUMMARY

The fine structure of normal rabbit and human blood leucocytes and rabbit lung macrophages was investigated. The use of phosphotungstic acid as a stain for glutaraldehyde fixed leucocytes provided much new information.

In an effort to determine the morphological changes associated with leucocyte activation to release endogenous pyrogen (EP), cells exposed to four different activating agents were compared with control cells not exposed to activators. Many fine structural differences between activated and control leucocytes were observed. However, EP was not visualized.

Metabolic inhibition of EP release by actinomycin-D in cells exposed to an activating agent did not result in blockade of any of the morphologic changes associated with activation. Thus, it appeared that no fine structural changes were intimately connected with EP release in leucocytes, although many changes occurred in the transformation to a general activation state. Several hypotheses concerning production and release of EP were discussed.

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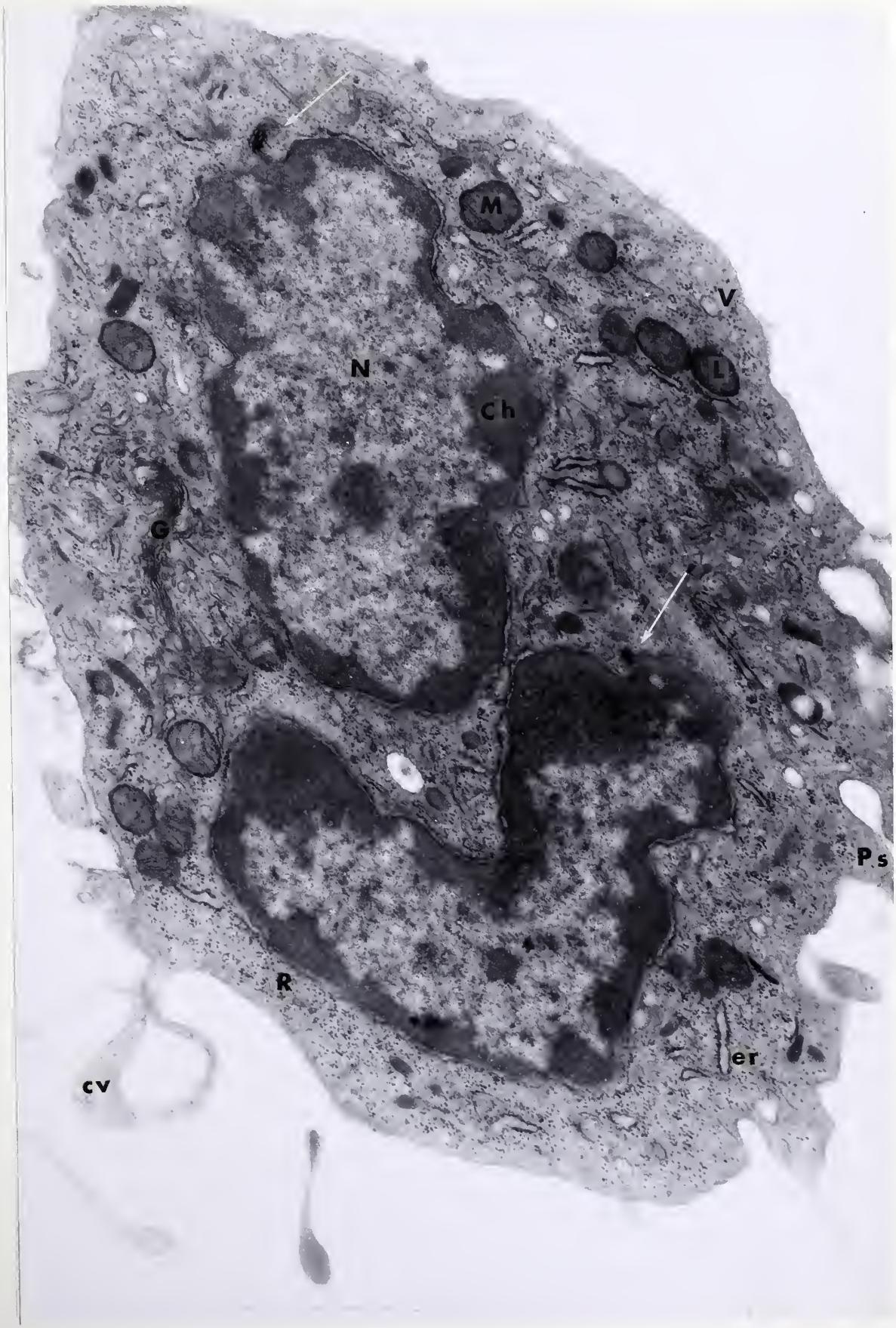
Figures

Figure 1

Normal human blood monocyte. The nucleus (N) is sectioned across its characteristic central indentation and therefore appears bilobed. The chromatin material (Ch) is arranged peripherally, subadjacent to the nuclear envelope which is composed of two parallel membranes. In certain areas, the external element of the nuclear envelope appears to undergo a form of specialization, characterized by whorls of membrane positioned in the juxtanuclear cytoplasm (see arrows). Within the cytoplasm, rough-surfaced endoplasmic reticulum (er), mitochondria (M), Golgi apparatus (G), lysosomes (L), pinocytic vesicles (V), and clusters of free ribosomes (R) can be seen. The cell surface is made irregular by the presence of pseudopodia (Ps) and cytoplasmic veils (cv). Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X36,000.


M
V
N
L
Ch
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Ps
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er
cv

Figure 2

Normal human blood lymphocyte. The chromatin material within the kidney-shaped nucleus occupies both central and peripheral locations. Whorls of membrane in the juxtanuclear area (arrows) appear to be specializations of the nuclear membrane. The cytoplasm contains the small, electron-dense free ribosomes, a few mitochondria, and a Golgi apparatus. A centriole (C) is also present. The cell surface has an irregular contour as a result of the presence of pseudopodia. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X41,000.

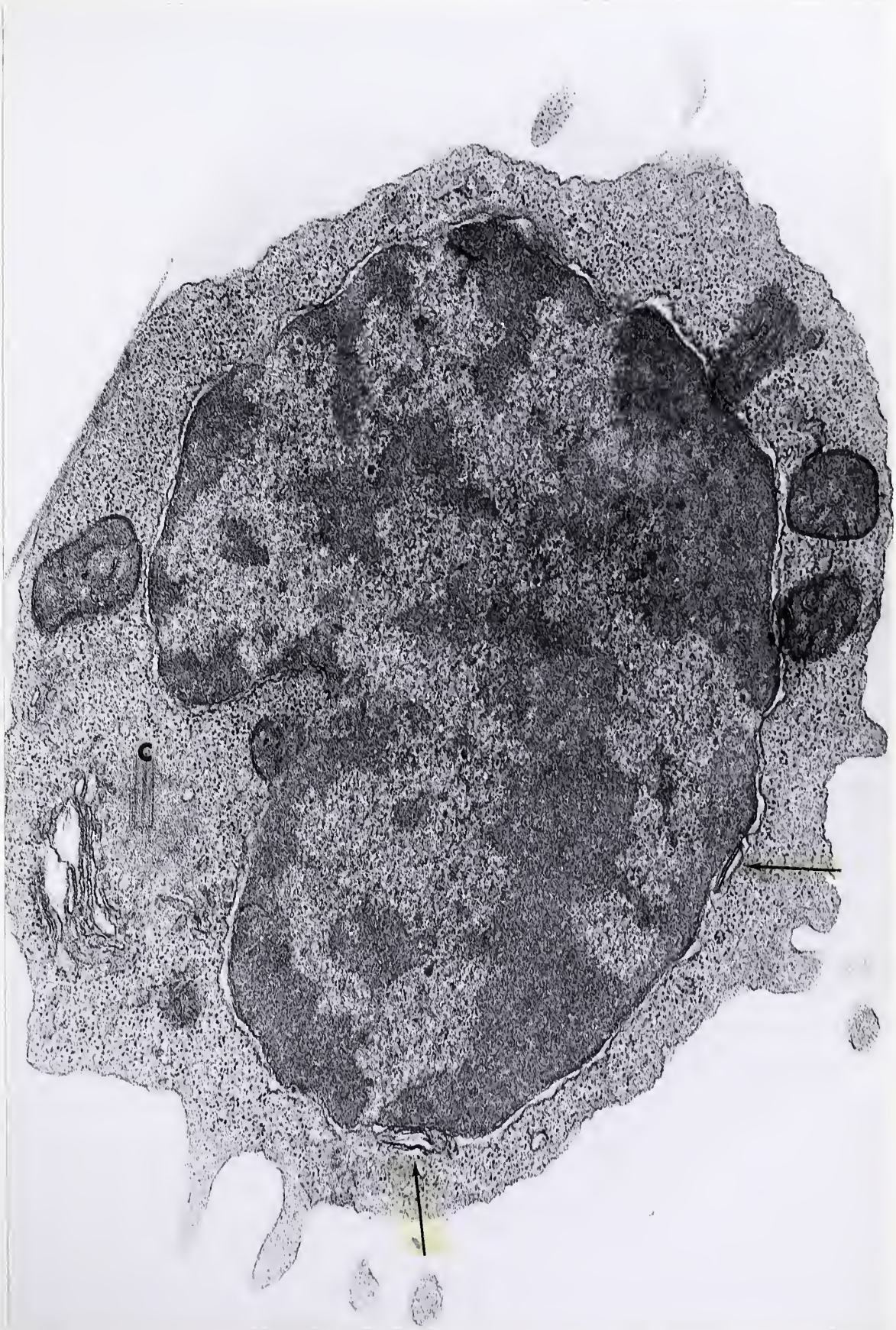

c

Figure 3

Normal human blood eosinophil. The bilobed nucleus contains peripherally arranged chromatin material. The skeins of dense, threadlike material seen within the nucleus of this cell were seen rarely in normal cells, but commonly in those cells treated with actinomycin-D (see Figures 40-43). The cytoplasm is filled with large, membrane-bound eosinophil granules (EG) which contain a dense, crystalline core material. A Golgi apparatus is seen centrally, and many small, irregular, membrane-bound vesicles are present. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X32,000.

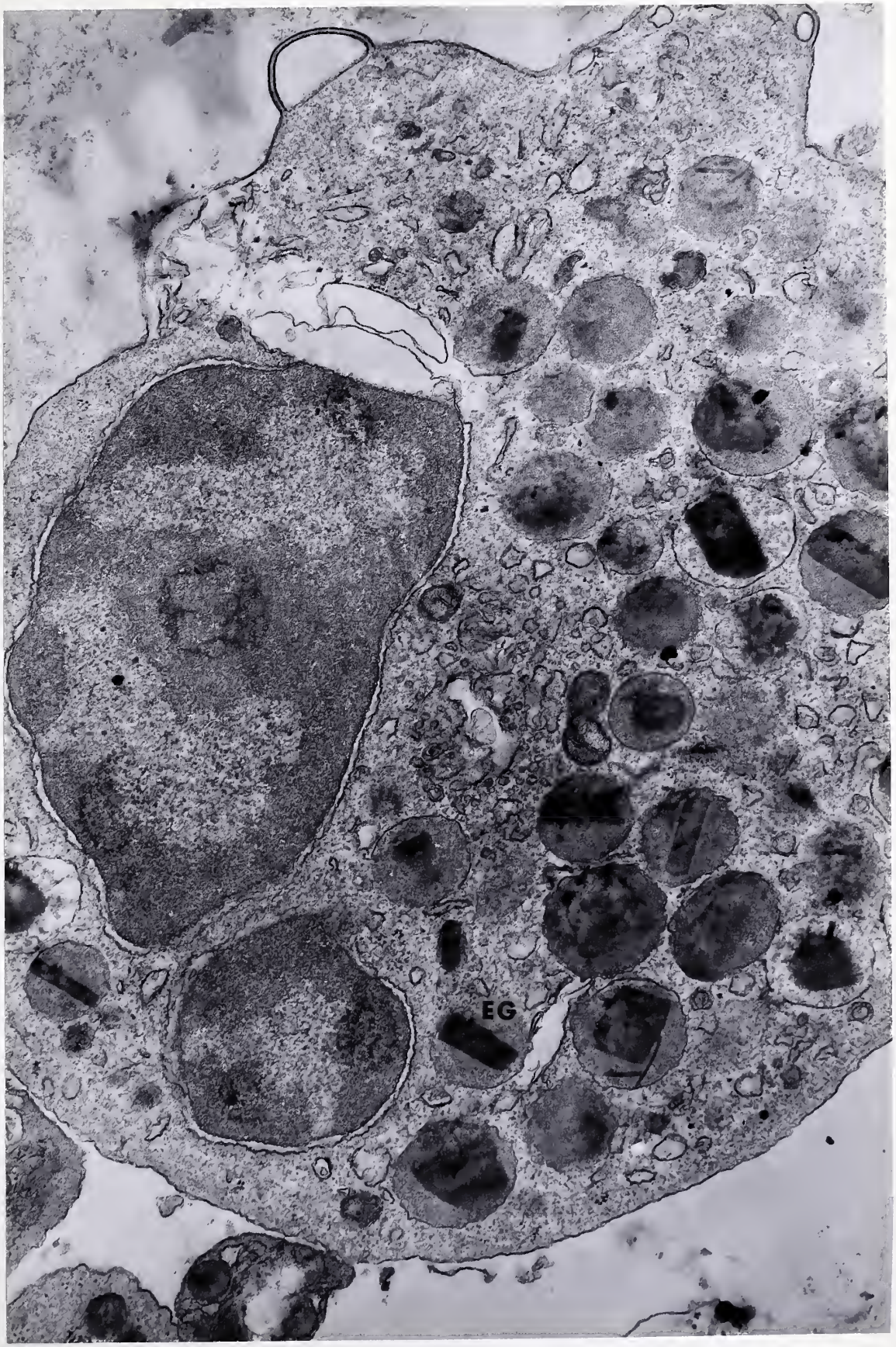

EG

Figure 4

Normal human PMN leucocyte. The multilobed nucleus contains cross-sectioned invaginations of cytoplasm which appear as islands within the nucleus. The chromatin material is not seen well. The cytoplasm contains numerous neutrophilic granules (Lysosomes) (L) containing amorphous material in a range of electron densities. Elements of endoplasmic reticulum (er) have wide cisternae, and a small Golgi apparatus can be seen (G). Small dense deposits of glycogen stain heavily in lead citrate and can be seen scattered throughout the cytoplasm. The cell surface is irregular because of the presence of many pseudopodia. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X51,000.

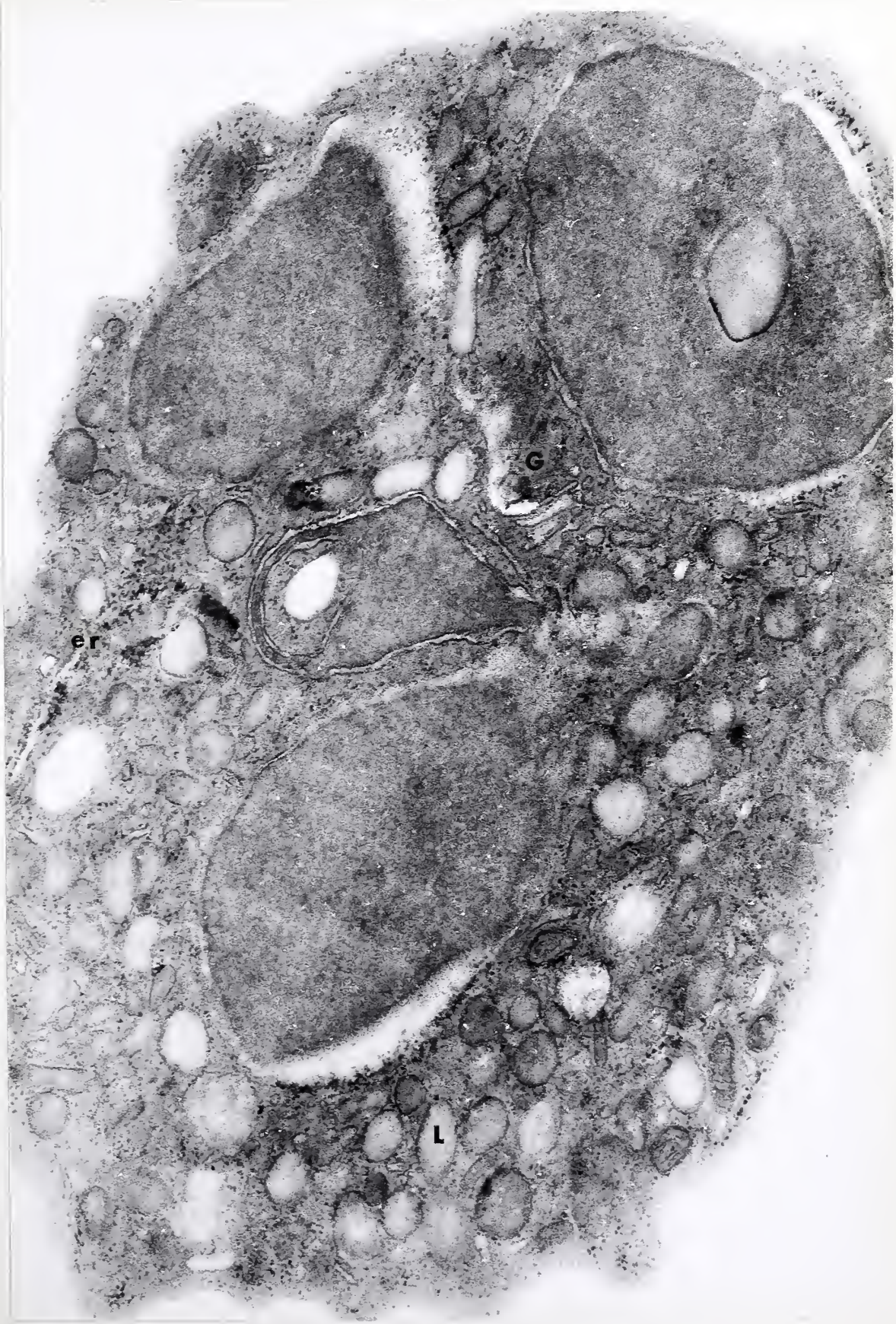

G
er
L

Figure 5

Normal human blood monocyte (stained with PTA). The nucleus is again sectioned across the deep central indentation. The chromatin material therein is arranged peripherally, and the finely granular nucleoplasm is centrally located. In certain regions, the chromatin is not apposed to the nuclear envelope (arrows). The cytoplasm in some of these regions has an increased density which appears to penetrate inside the nucleus; in others, the cistern of the nuclear envelope is increased in density. These areas are thought to represent pores in the nuclear envelope. Within the cytoplasm, the mitochondria appear striped because the cristae and membranes do not stain whereas the matrix does. Lysosomes (L) stain densely, and the ground hyaloplasm is finely granular. Fixation in glutaraldehyde. PTA stain. X45,000.

L

Figure 6

Normal human blood eosinophil (stained with PTA). The nucleus contains peripherally arranged chromatin material interrupted at intervals similar to those seen in monocytes. These areas are thought to represent nuclear pores. The eosinophil granules, in contrast to their appearance in osmium-fixed material, contain lucent, angular core material surrounded by a dense, amorphous peripheral substance. Fixation in glutaraldehyde. PTA stain. X41,000.

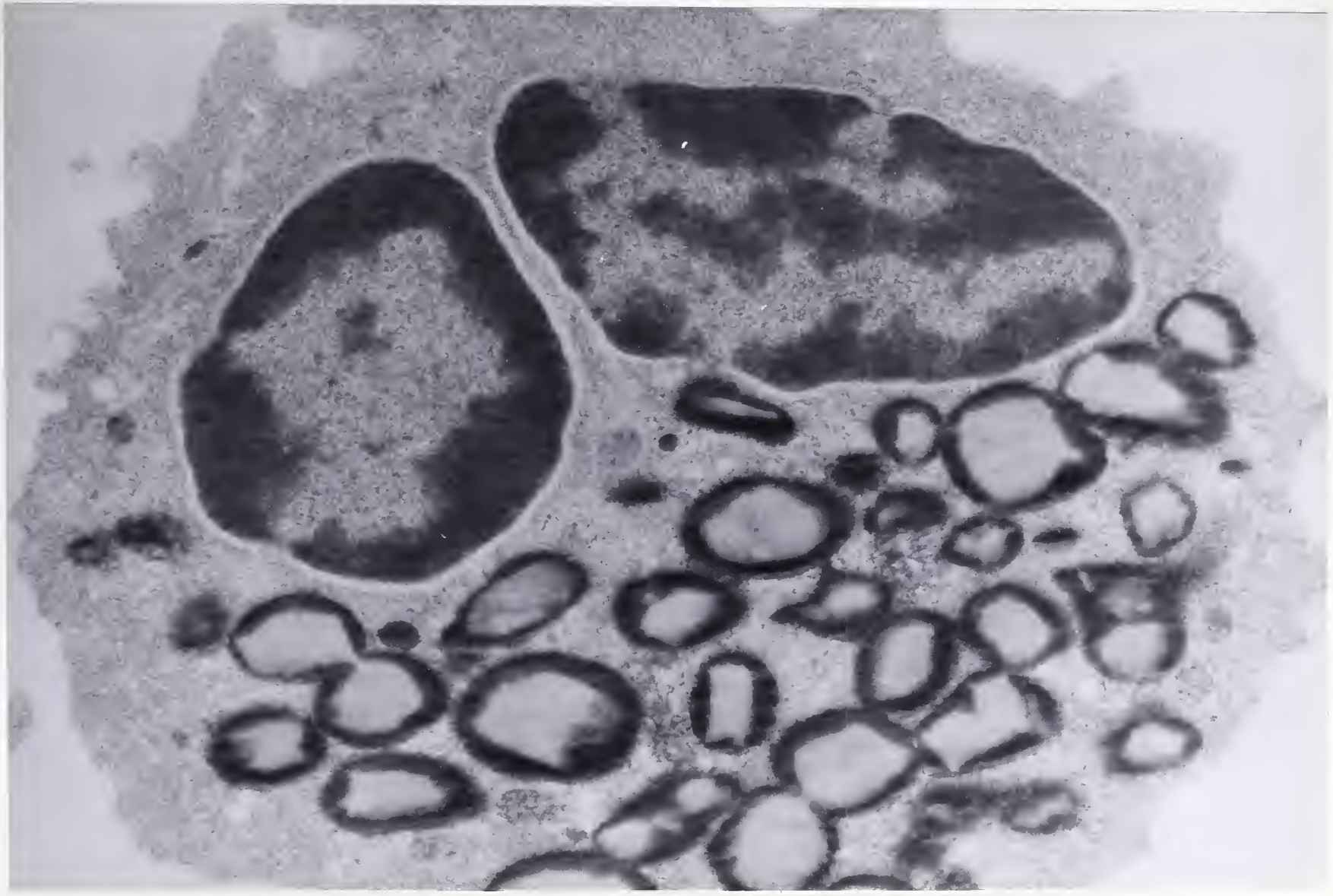


Figure 7

Normal human blood PMN leucocyte (stained with PTA). In contrast with the nuclei of the other leucocytes stained with PTA, the PMN leucocyte nucleus contains centrally clumped chromatin material. Only a narrow fringe of chromatin density is apposed without interruption to the nuclear envelope. No areas resembling the nuclear pores of monocytes or eosinophils can be seen. The nucleoplasm appears electron-lucent and without fine granularity. The cytoplasm contains a variety of organelles, only some of which could be identified with certainty. The large, dense round to oval bodies are PMN leucocyte granules (lysosomes) (L). The larger structures with dense amorphous material apposed to the limiting membrane are probably digestive vacuoles (DV). The area of alternating density and lucency is probably a Golgi apparatus (G). The other organelles may be smaller lysosomes, pinocytic vesicles, or phagocytic vacuoles. The dense structures whose length exceeded width by 10 times or more appeared to have no correlate in osmium fixed material, and may represent stained cisternae of endoplasmic reticulum (arrows). The ground hyaloplasm was dense and finely granular, and focal areas of increased density can be seen near the plasma membrane. Fixation in glutaraldehyde. PTA stain. X41,000.

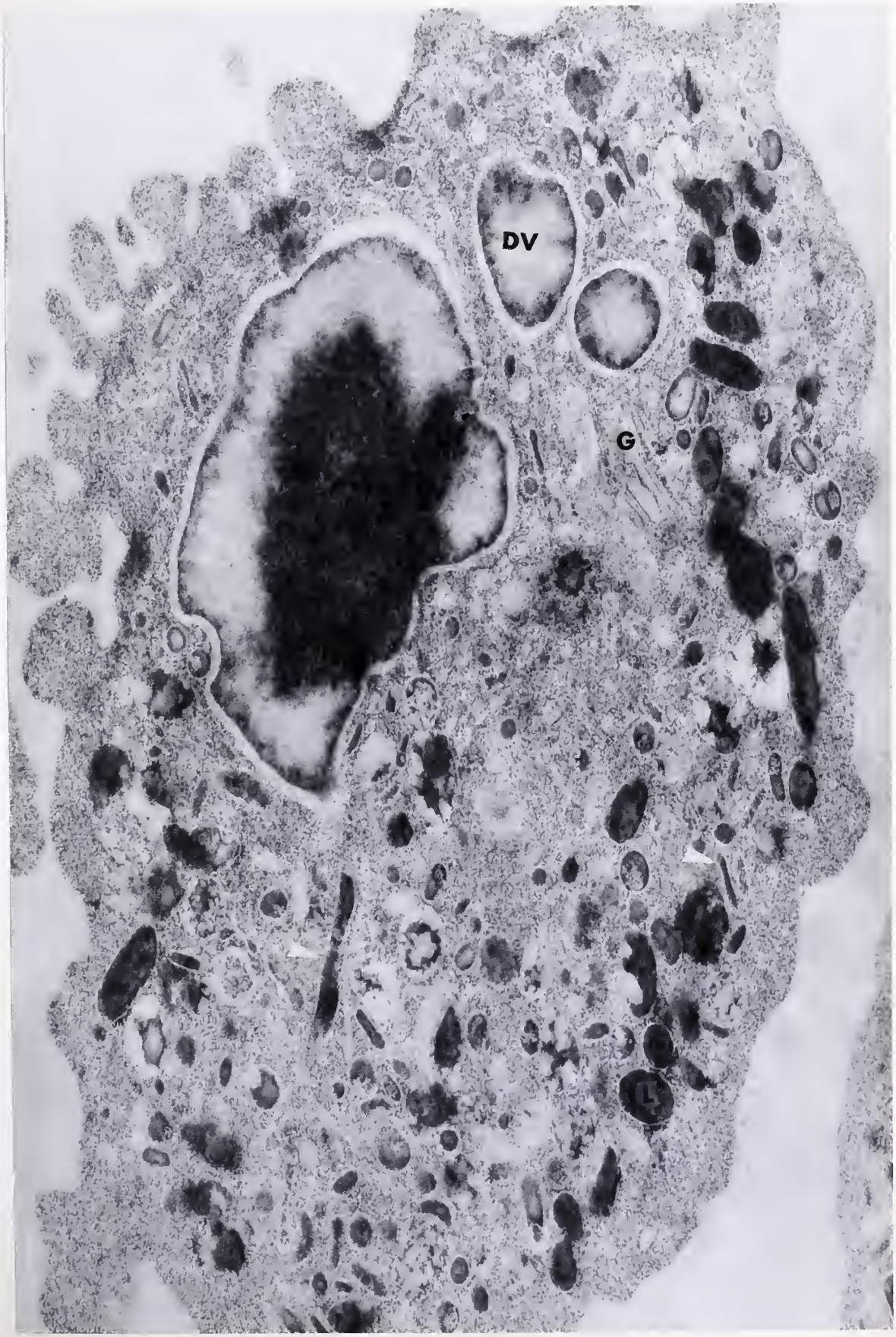

DV
G
L

Figure 8

Normal rabbit blood monocyte. This cell appears similar to the human monocyte in many respects. The large nucleus with central invagination is bordered by a nuclear envelope. Whorls of membrane in the juxta-nuclear cytoplasm in relation to the nuclear envelope can be seen. The cytoplasm contains much endoplasmic reticulum, many mitochondria, many pinocytic vesicles, a few lysosomes, and an abundance of free ribosomes. The cell surface is irregular, and a number of cyto-plasmic veils are seen. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X35,000.

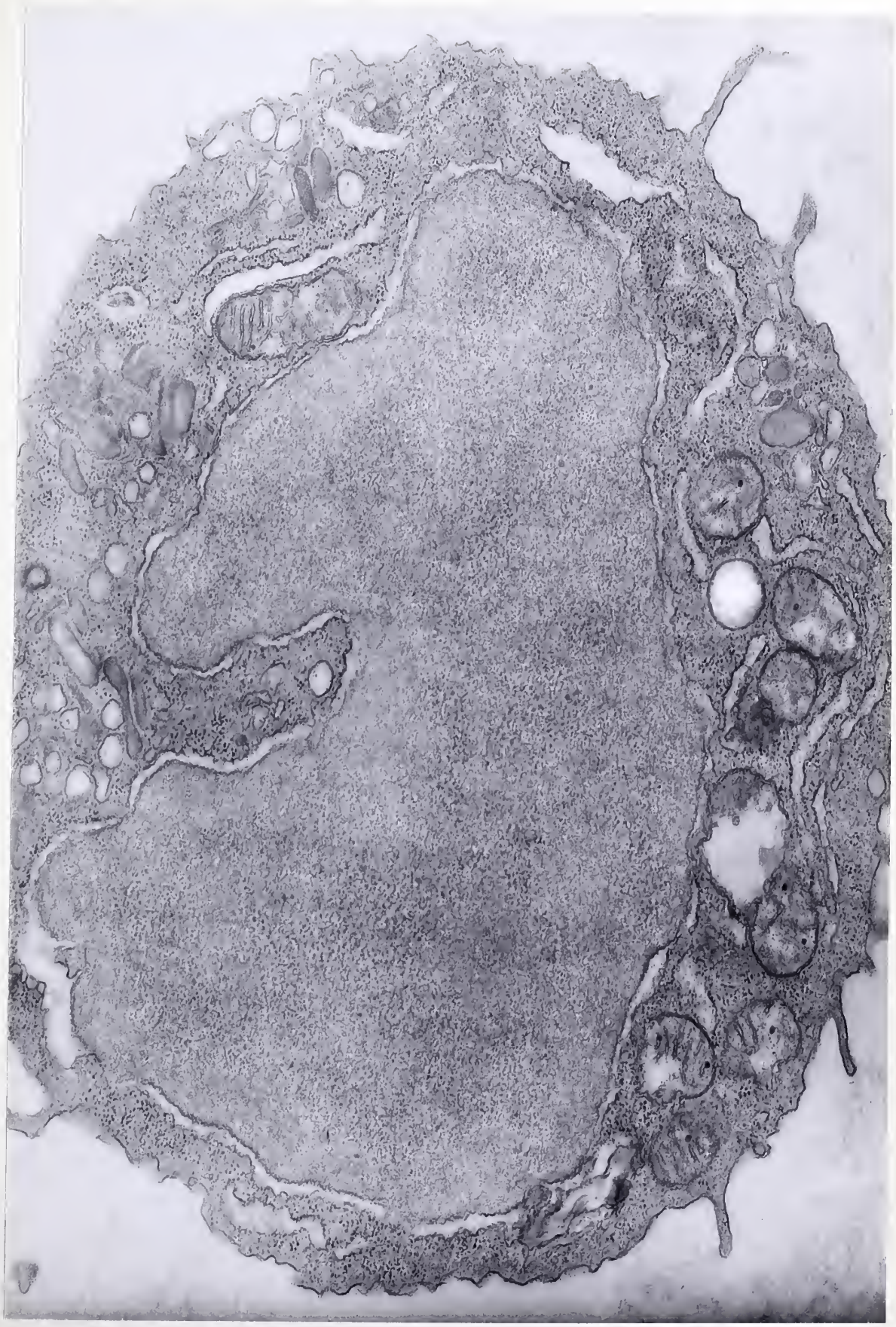


Figure 9

Normal rabbit blood lymphocyte. Also similar to its human counterpart, this cell has a large nucleus and thin rim of cytoplasm. A Golgi apparatus, a few large mitochondria, and many free ribosomes are evident. The area of the Golgi apparatus is seen in Figure 10 under higher magnification. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X33,000.

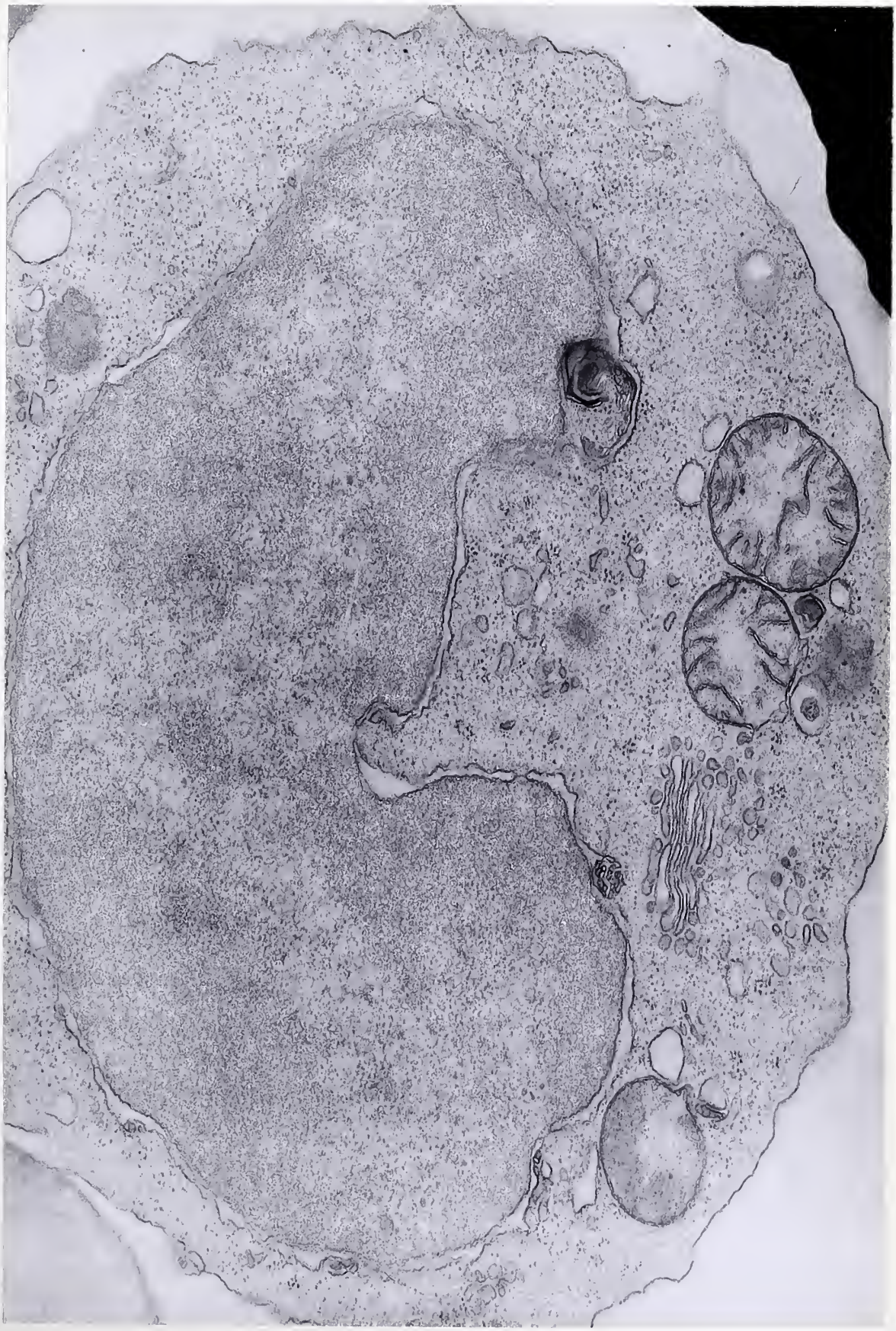
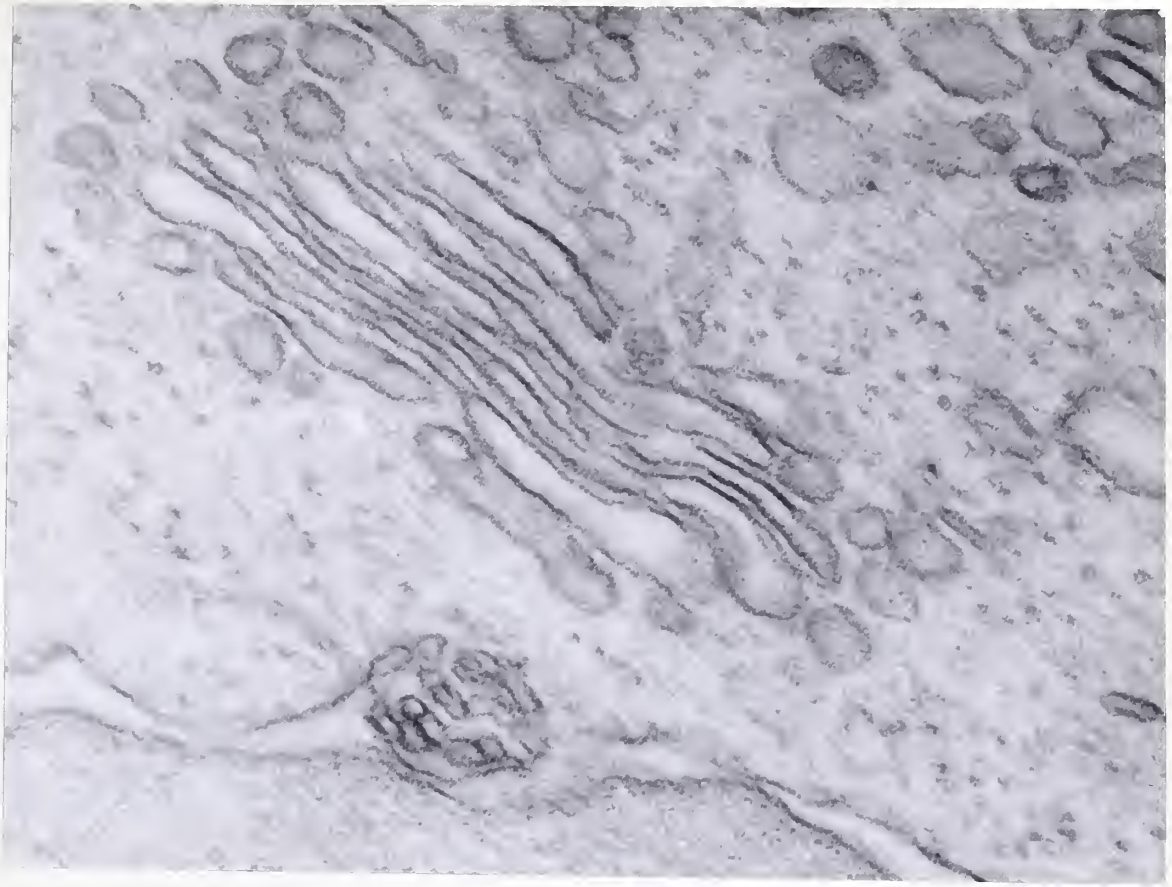


Figure 10

Normal rabbit blood lymphocyte. In the lower part of this micrograph, an area of nucleus with double-layered nuclear envelope can be seen. Whorls and reduplications of tubular membrane in the juxtanuclear cytoplasm involve the outer layer of the nuclear envelope, but the inner layer is separate. Some of these membranes appear denser than others. The lamellae and vesicles of the Golgi apparatus occupy the greater part of this micrograph, and appear discontinuous with the specialization of the nuclear envelope. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X118,000.

Figure 11

Normal rabbit blood eosinophil. The nucleus is sectioned through three lobes. No reduplications of the external element of the nuclear envelope can be identified in this cell, though they were common in other rabbit eosinophils. The characteristic eosinophil granules are quite different from their human counterparts. Their entire volume appears filled by a dense, amorphous material over which a still denser, crystalline material is superimposed. The other cytoplasmic structures (mitochondria, pinocytic vesicles, endoplasmic reticulum, and glycogen deposits) are similar to those found in normal human blood eosinophils. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X26,000.



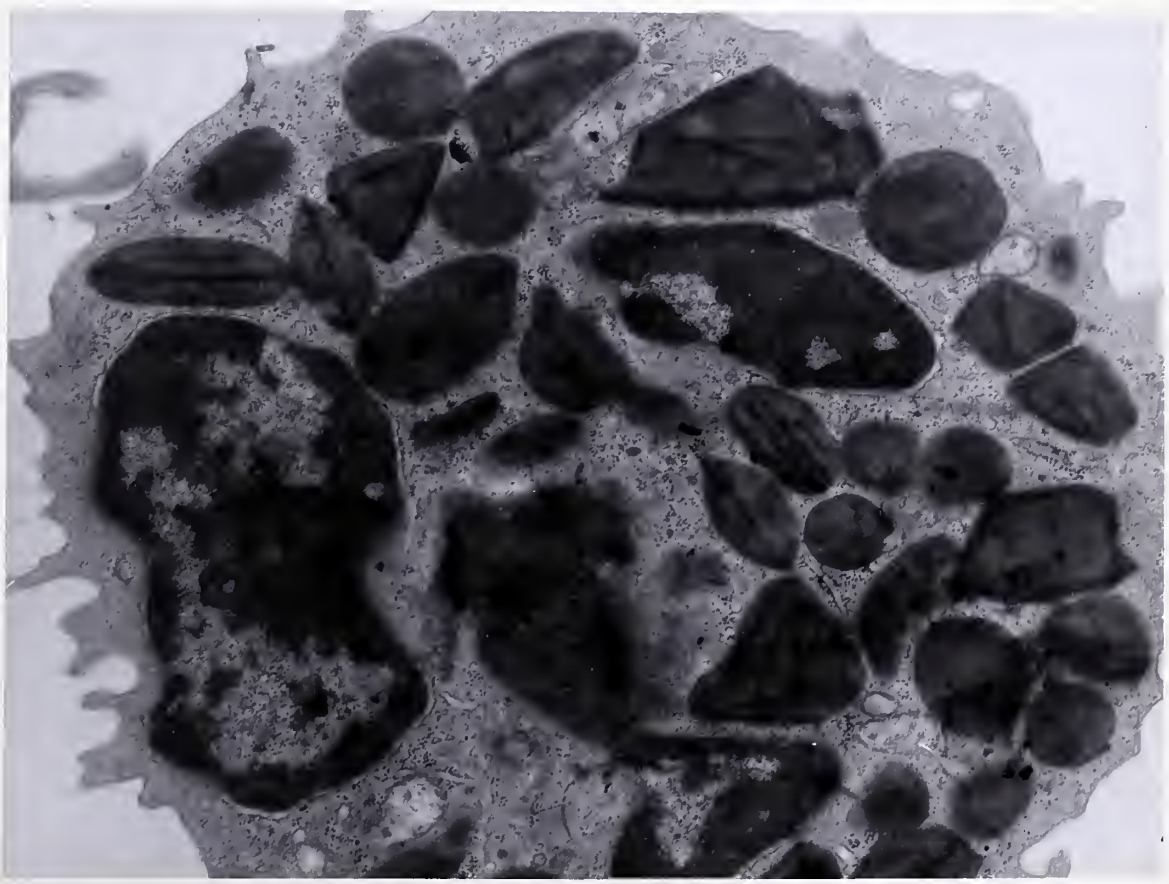


Figure 12

Normal rabbit blood PMN leucocyte. The multilobed nucleus encloses a peripherally arranged chromatin material, interrupted in apposition to the nuclear envelope. Although the membranes of the nuclear envelope are seen indistinctly in cells with brief exposure to osmium tetroxide, the cistern of the envelope contains material similar to nucleoplasm in the areas of suspected nuclear pores (arrows). In the cytoplasm, there appear to be two types of membrane bound granules (lysosomes). The larger type is more refractory to thin sectioning than the smaller, more numerous type. A few small mitochondria are present, and many dense deposits of glycogen are scattered through the cytoplasm. The cell surface is irregular with many pseudopodia. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X36,000.

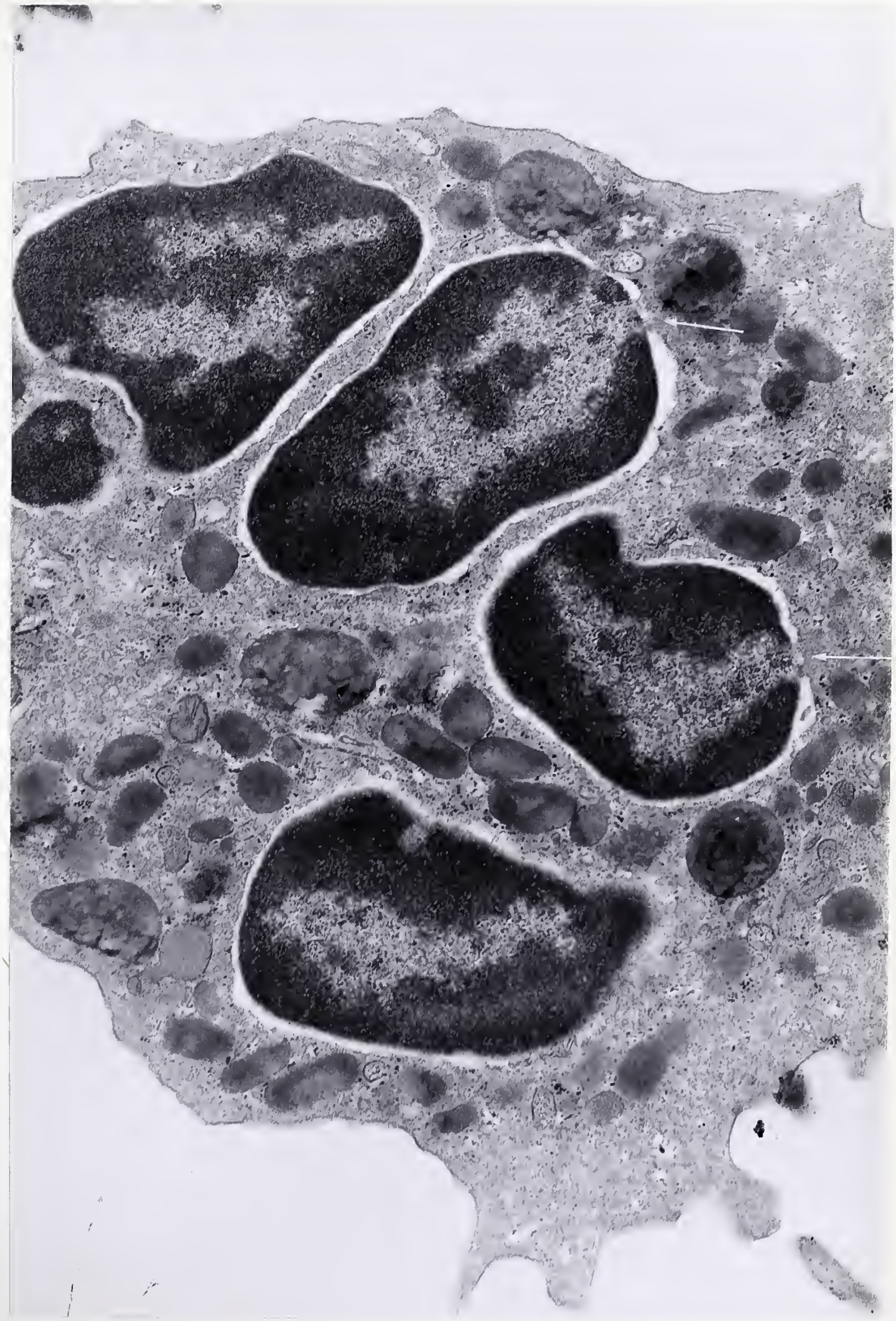


Figure 13

Normal rabbit lung macrophage. An extensive cytoplasmic invagination is present in the center of the large nucleus in this section. A considerable amount of endoplasmic reticulum with adherent ribosomes is present in the cytoplasm, along with dense lysosomes of differing shape, mitochondria, and some digestive vacuoles. Many small membrane bound vesicles are present, and the cell surface is extremely active with many pseudopodia and cytoplasmic veils. Over 95% of cells seen in washings of rabbit bronchoalveolar tree were of this type. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate and uranyl acetate stain. X31,000.

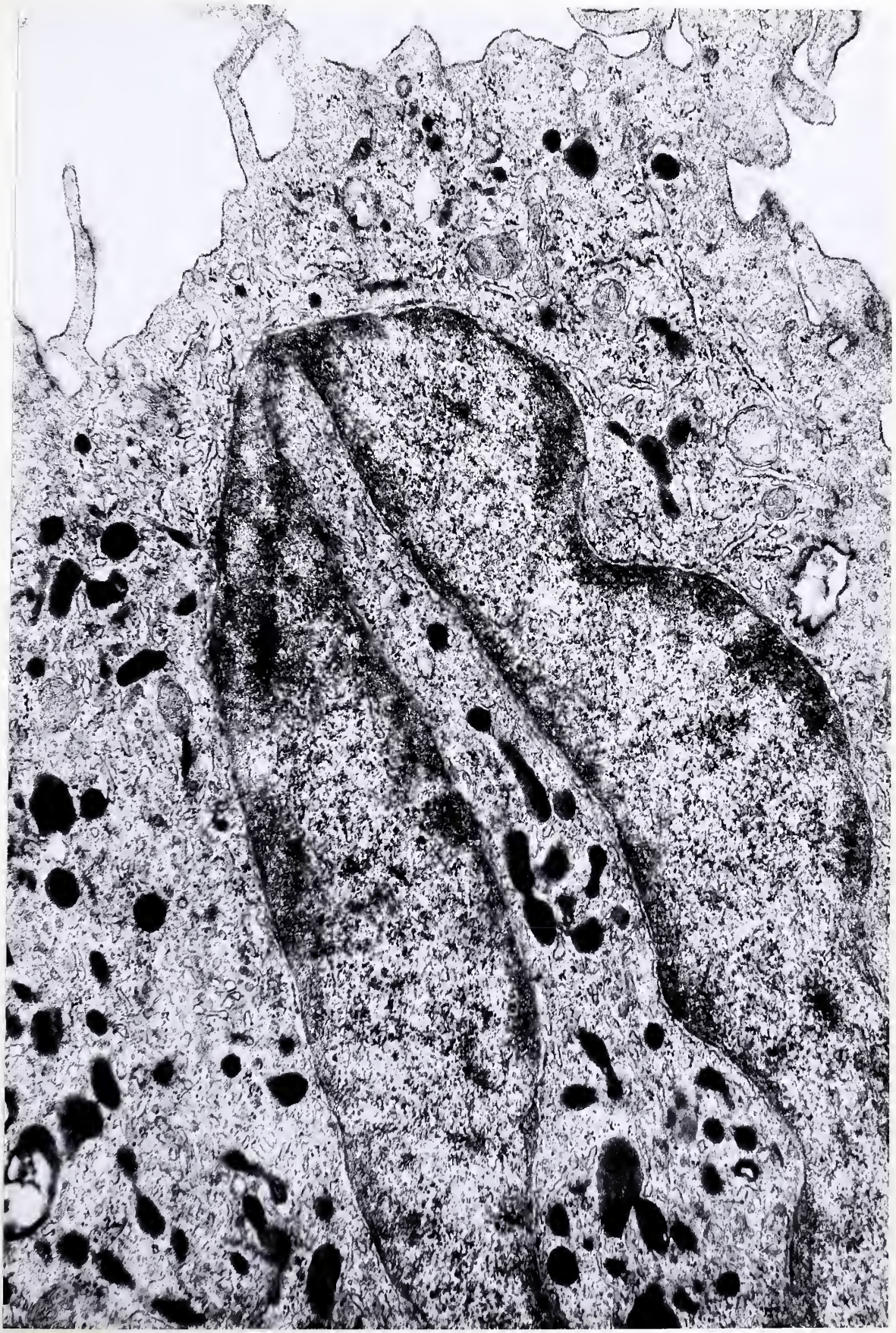
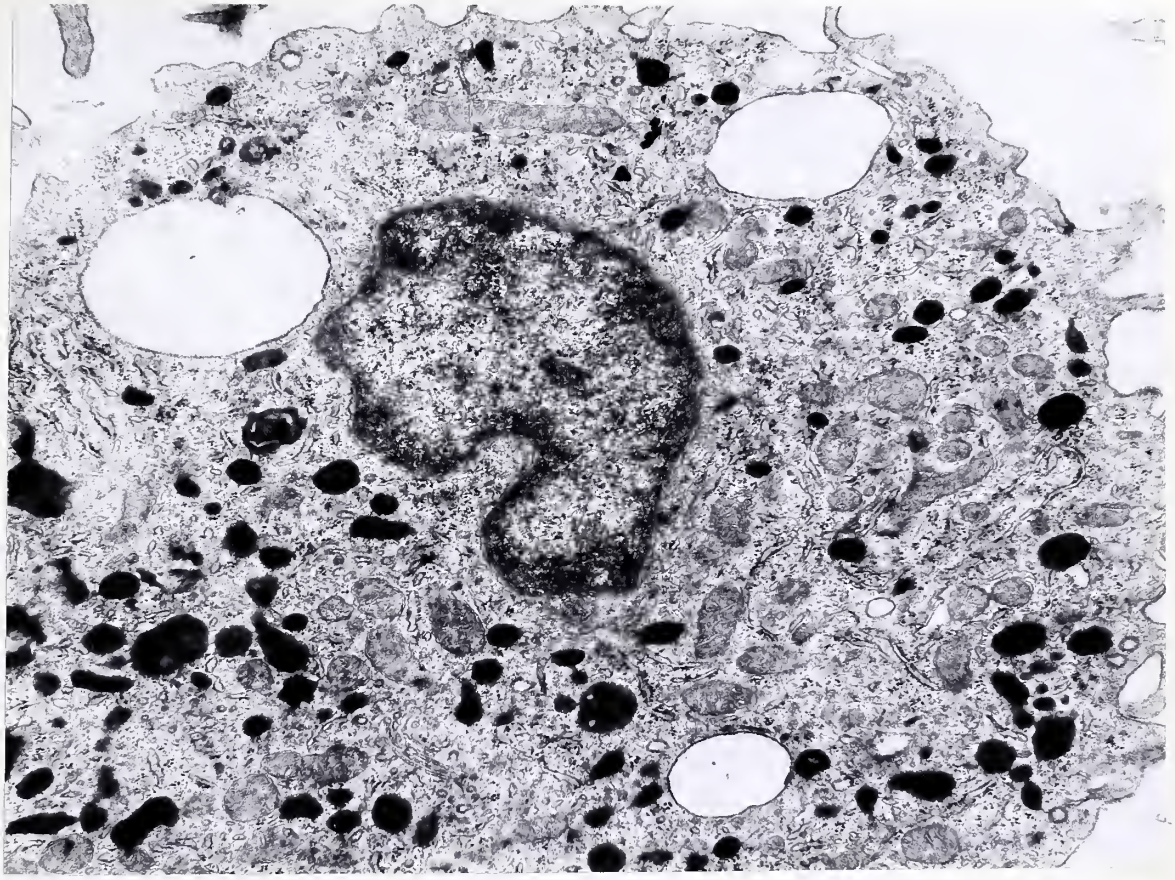


Figure 14

Normal rabbit lung macrophage. Three large lucent digestive vacuoles are present; otherwise the cell is similar to that shown in Figure 13. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate and uranyl acetate stain. X21,000.

Figure 15

Normal rabbit lung macrophage. This high magnification view of cross-sectioned cytoplasmic veils demonstrates the fine, fibrillar mucoid substance coating the cell surface and extending into the extracellular substance. No cytoplasmic organelles are found within the narrow cytoplasmic veils. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate and uranyl acetate stain. X175,000.



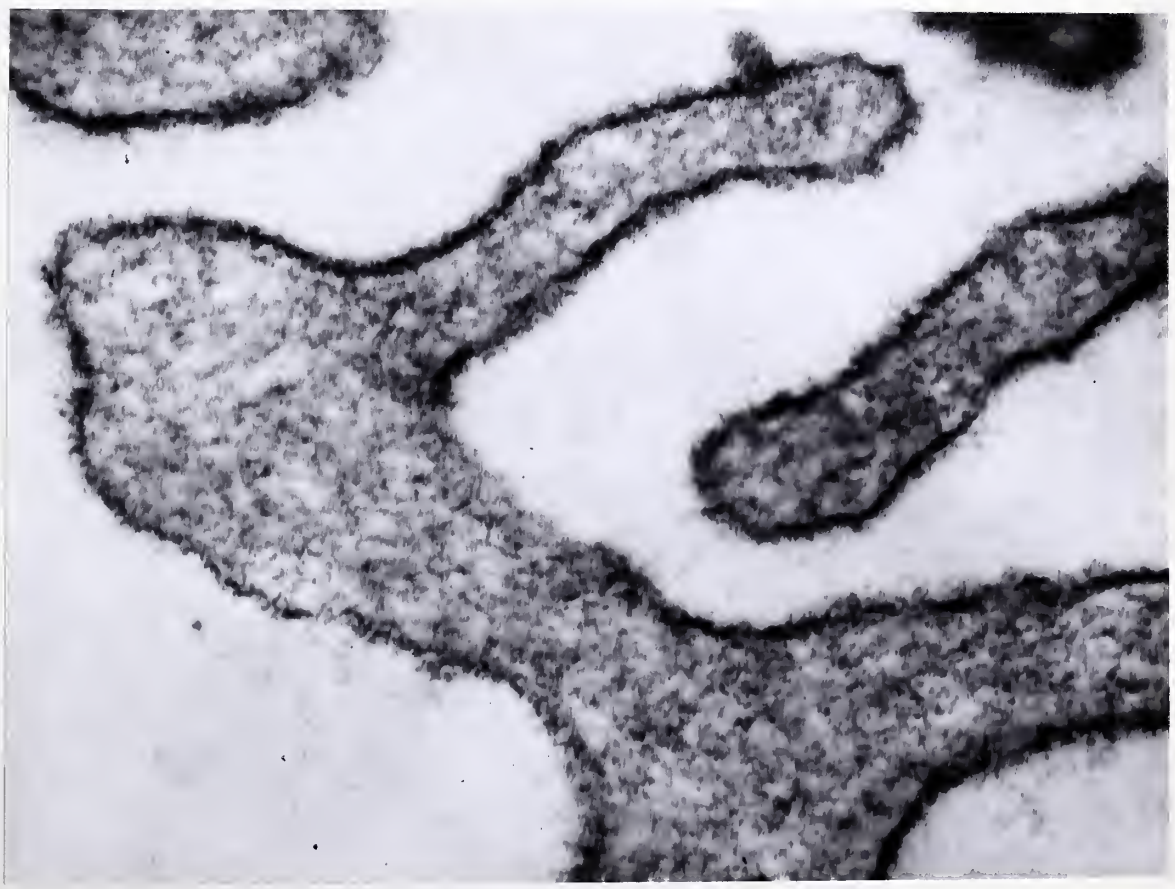


Figure 16

Rabbit peritoneal exudate PMN leucocyte. In this cell, two types of granules are visible, and a large Golgi apparatus is present. A skein of threadlike density is present in the nuclear lobe at the left, and an adjacent cytoplasmic invagination containing a row of granules can be seen. Explanation of the differences between this cell and the normal rabbit blood PMN leucocyte can be found on the next page. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X36,000.

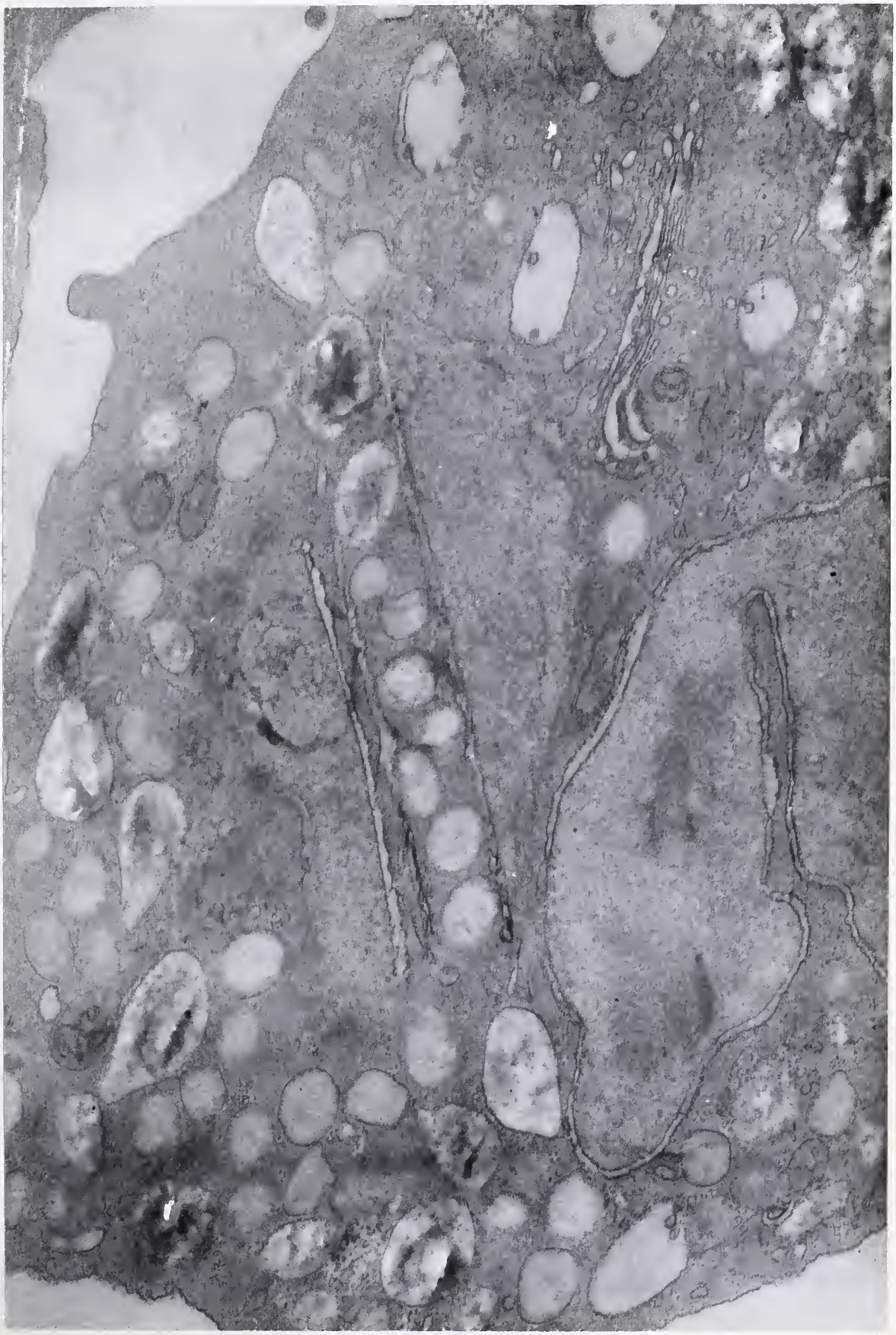


Figure 17 (above)

Normal rabbit blood PMN leucocyte. Briefer fixation than that of the cell in Figure 12. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X26,000.

Figure 18 (below)

Rabbit peritoneal exudate PMN leucocyte. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X24,000.

The exudate cells which are releasing EP, exemplified by Figures 16 and 18, differ from the control normal blood PMN leucocytes seen in Figures 17 and 12 in several consistently observed ways. The exudate cells have a more irregular border because more pseudopodia are present. Many digestive vacuoles containing the endogenous glycogen used to induce the formation of the peritoneal exudate are seen in exudate cells, but are absent from controls. On the other hand, deposits of endogenous cytoplasmic glycogen are less abundant in exudate cells. Elements of endoplasmic reticulum and Golgi apparatus are more frequently encountered in exudate cells. Lysosomes of both types seemed more common in exudate cells, although the relative proportions of large and small lysosomes was similar in exudate and control cells. In addition, exudate cells were considerably less refractory to thin sectioning than controls after comparable fixations.

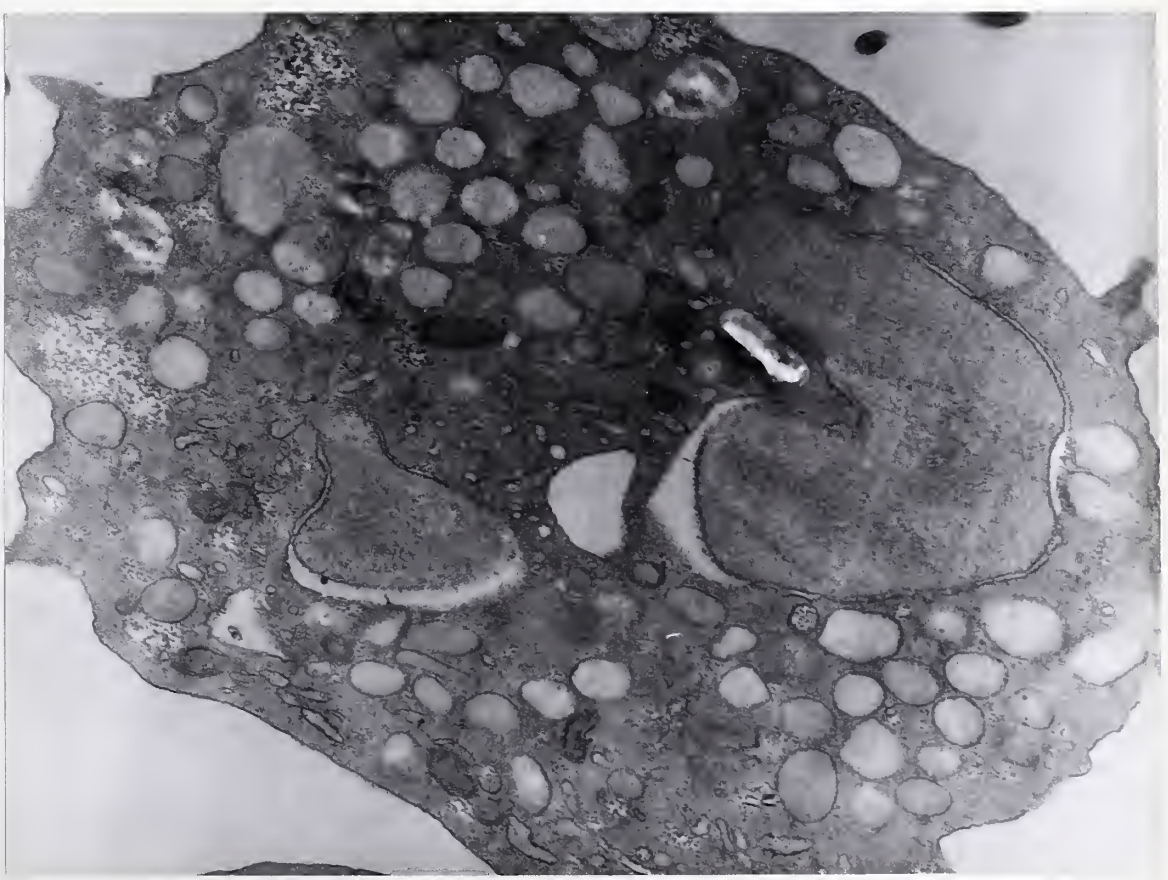


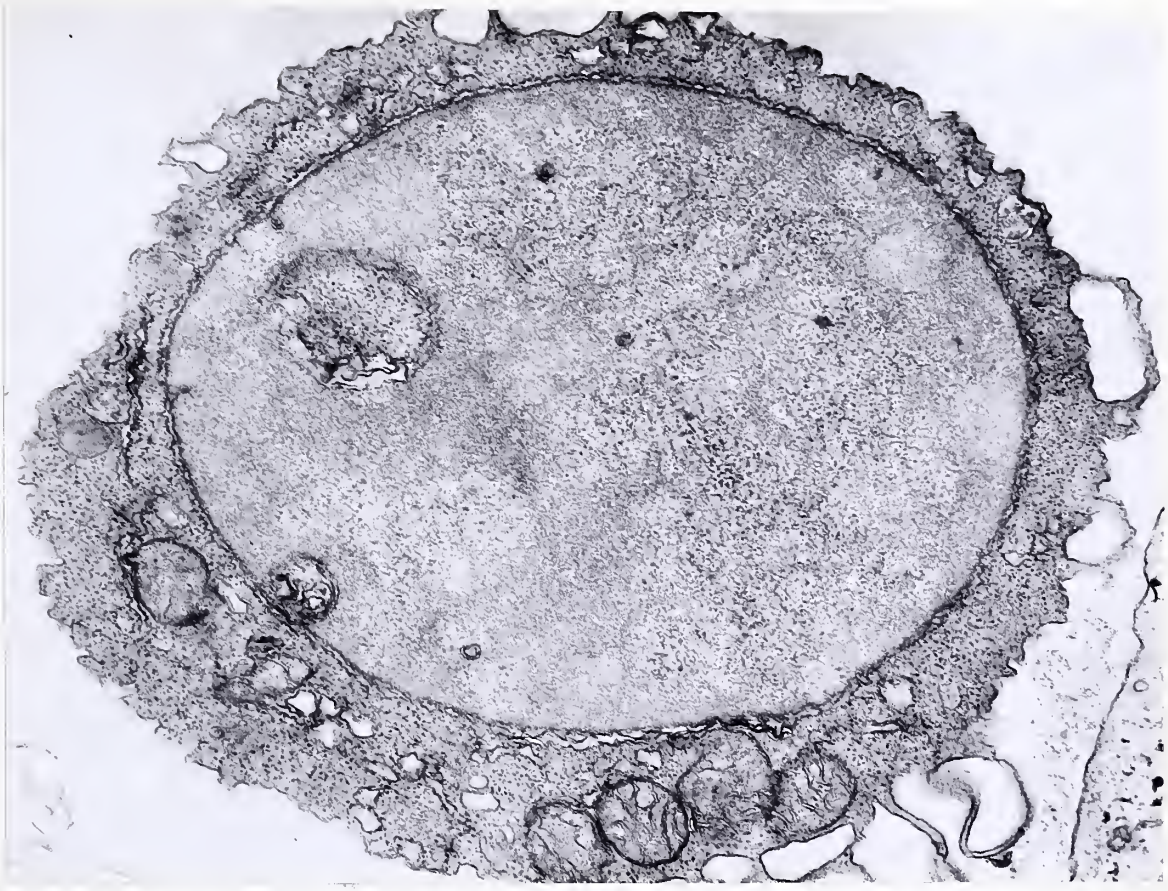
Figure 19

Control human lymphocyte. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X32,000.

Figure 20

Etiocholanolone-treated human lymphocyte. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X30,000.

The lymphocyte treated with etiocholanolone is not different in any consistent way from the control lymphocyte. Both cells have an active cell surface with many cytoplasmic veils. Perinuclear whorls of membrane are seen with similar frequency. Cytoplasmic organelles are similar in type, number, and distribution.



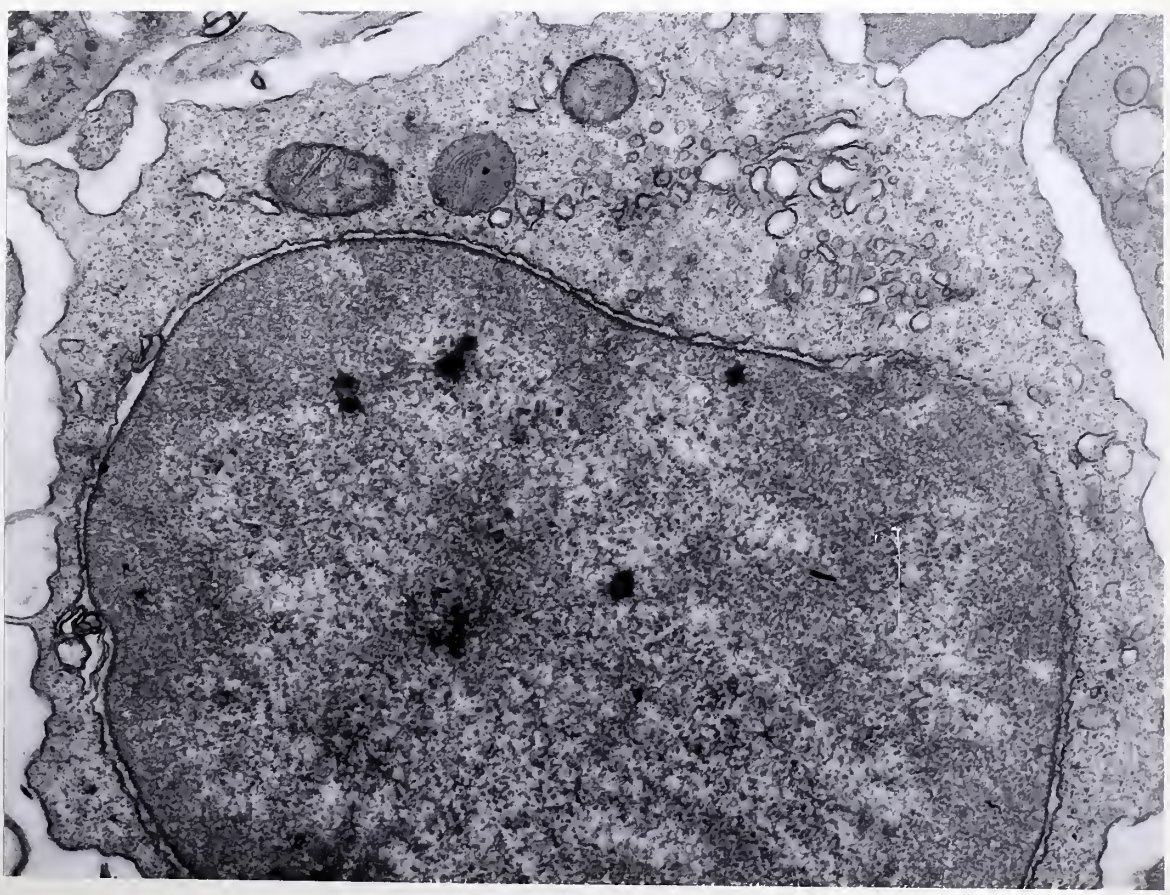


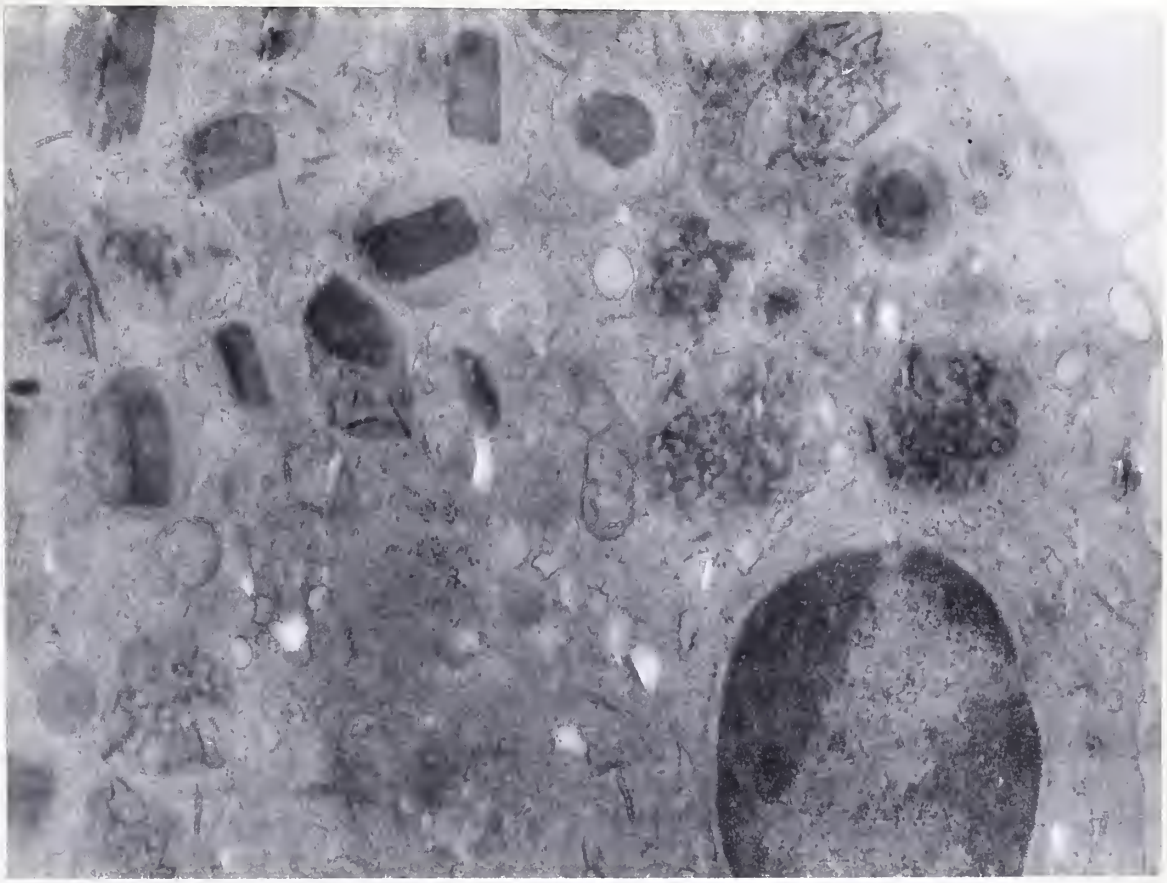
Figure 21

Control human eosinophil. Reference to Figure 3 will demonstrate that the fragmentation of the crystalline material seen in some of the granules of this cell is not a typical finding amoung control cells. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X33,000.

Figure 22

Etiocholanolone-treated human eosinophil. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X35,000.

The only consistent difference observed between etiocholanolone treated eosinophils and control eosinophils was a more diffuse chromatin distribution in the cell incubated with etiocholanolone. Eosinophils were, however, encountered only rarely in sections from both preparations.



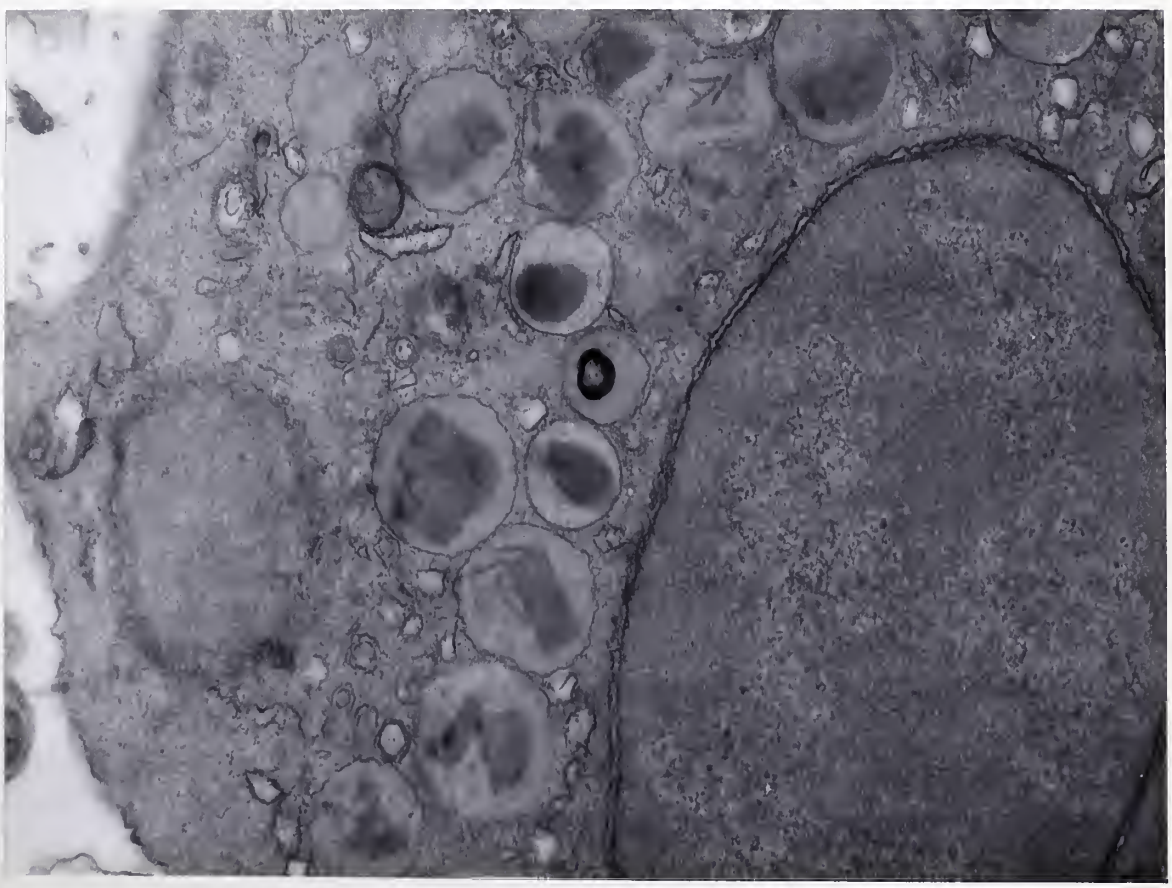


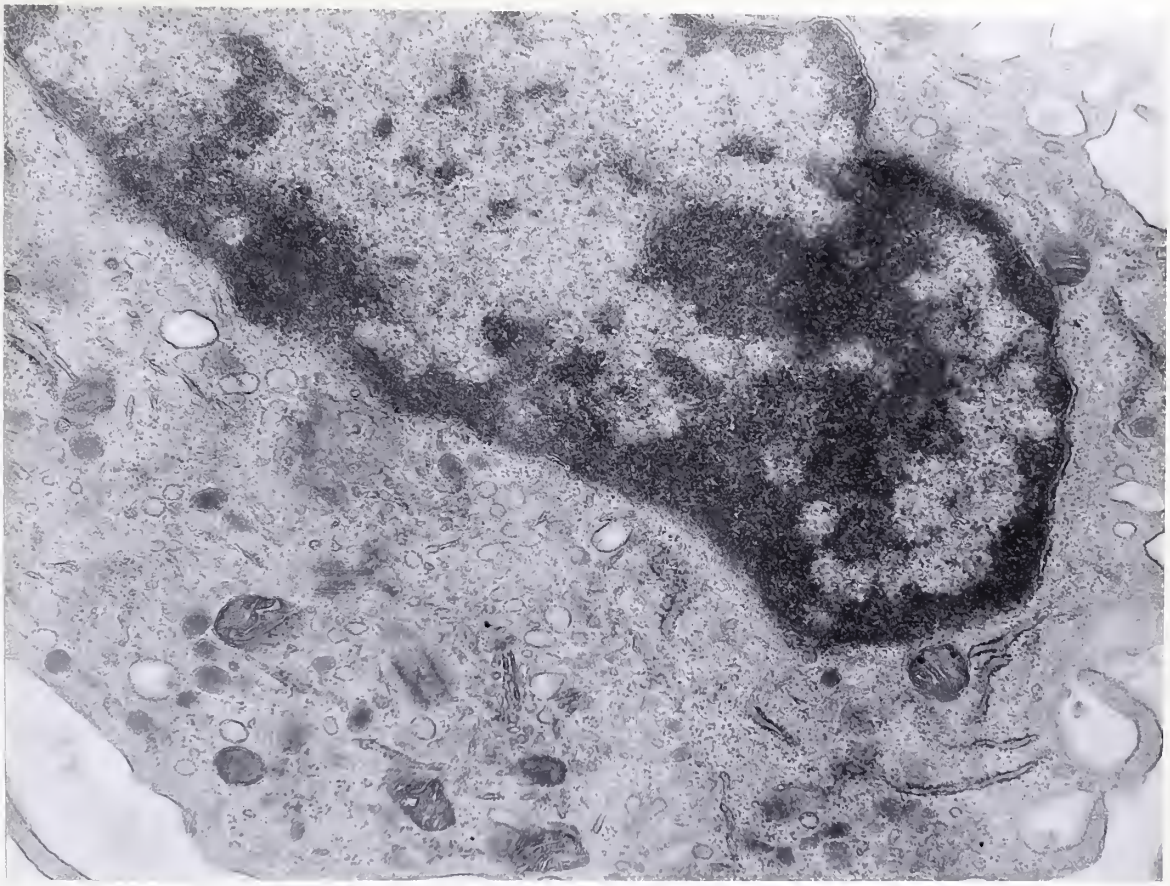
Figure 23

Control human monocyte. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X25,000.

Figure 24

Etiocholanolone-treated human monocyte. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X30,000.

The monocyte incubated with etiocholanolone has been implicated in the release of EP. The chromatin distribution of the etio-treated cells is less condensed than that of the control cell. Many more perinuclear whorls of membrane are seen in the etio-treated cell, and where present occupy a greater area. In the cytoplasm, the mitochondria of the etio-treated cell are larger with less closely packed cristae than those of the control cell. Elements of endoplasmic reticulum are more abundant in the etio-treated monocyte, and are often seen in stacks of parallel double membranes as in the lower left of Figure 24. The prevalence of lamellae and vesicles of the Golgi apparatus in sections of etio-treated cells appeared greater. No difference in populations of lysosomes, small vesicles, or free ribosomes was evident. The ground hyaloplasm was less dense in the etio-treated monocyte.



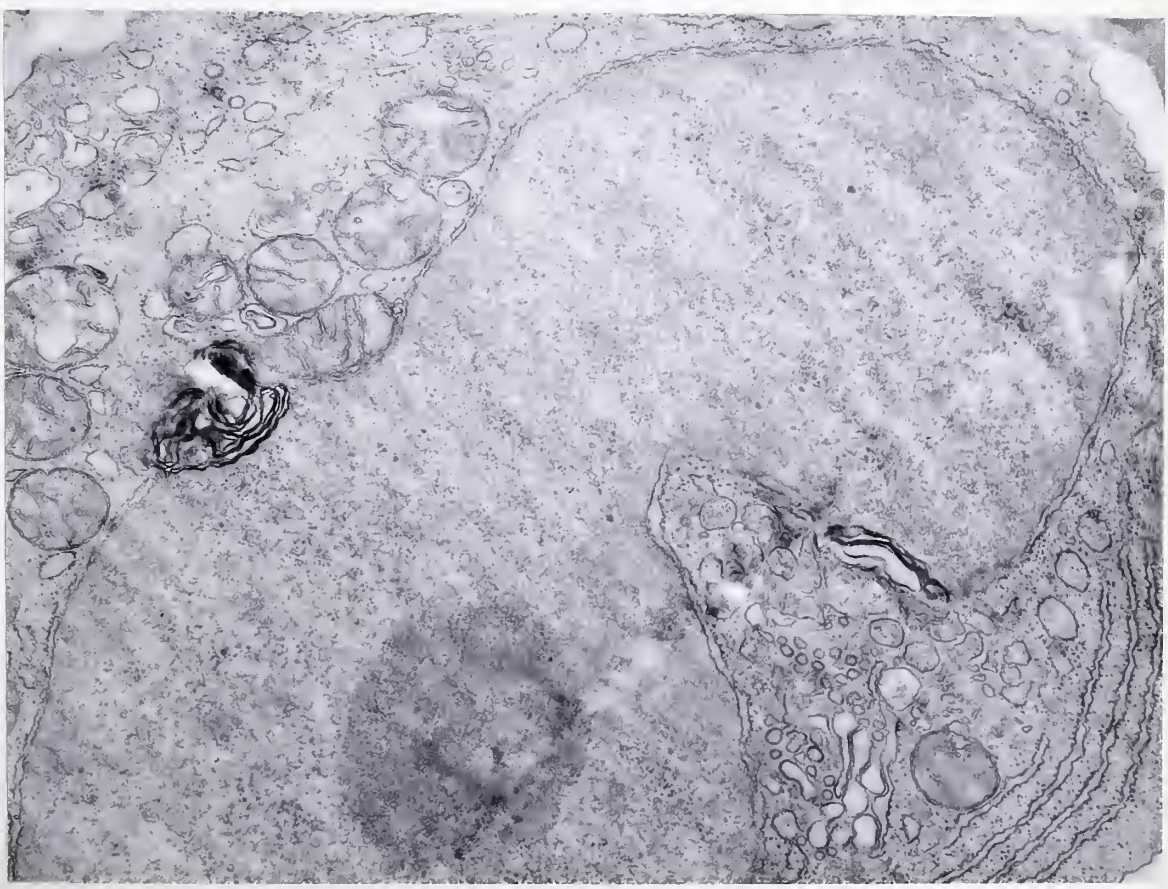


Figure 25

Etiocholanolone-treated human PMN leucocyte. This cell is sectioned near the cell surface; therefore no nucleus is seen. Of note is the profusion of cytoplasmic veils at the cell surface, arising from a narrow base of cytoplasm. The veils are coated by a fine mucoid substance similar to that seen on the surface of normal rabbit lung macrophages. In many areas, two adjacent veils appear in the process of fusion, enclosing a volume of extracellular substance. In other areas, this material appears either partially or fully incorporated into a cytoplasmic vacuole. Explanation of the differences between etiocholanolone-treated and control PMN leucocytes can be found on the following page. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X50,000.

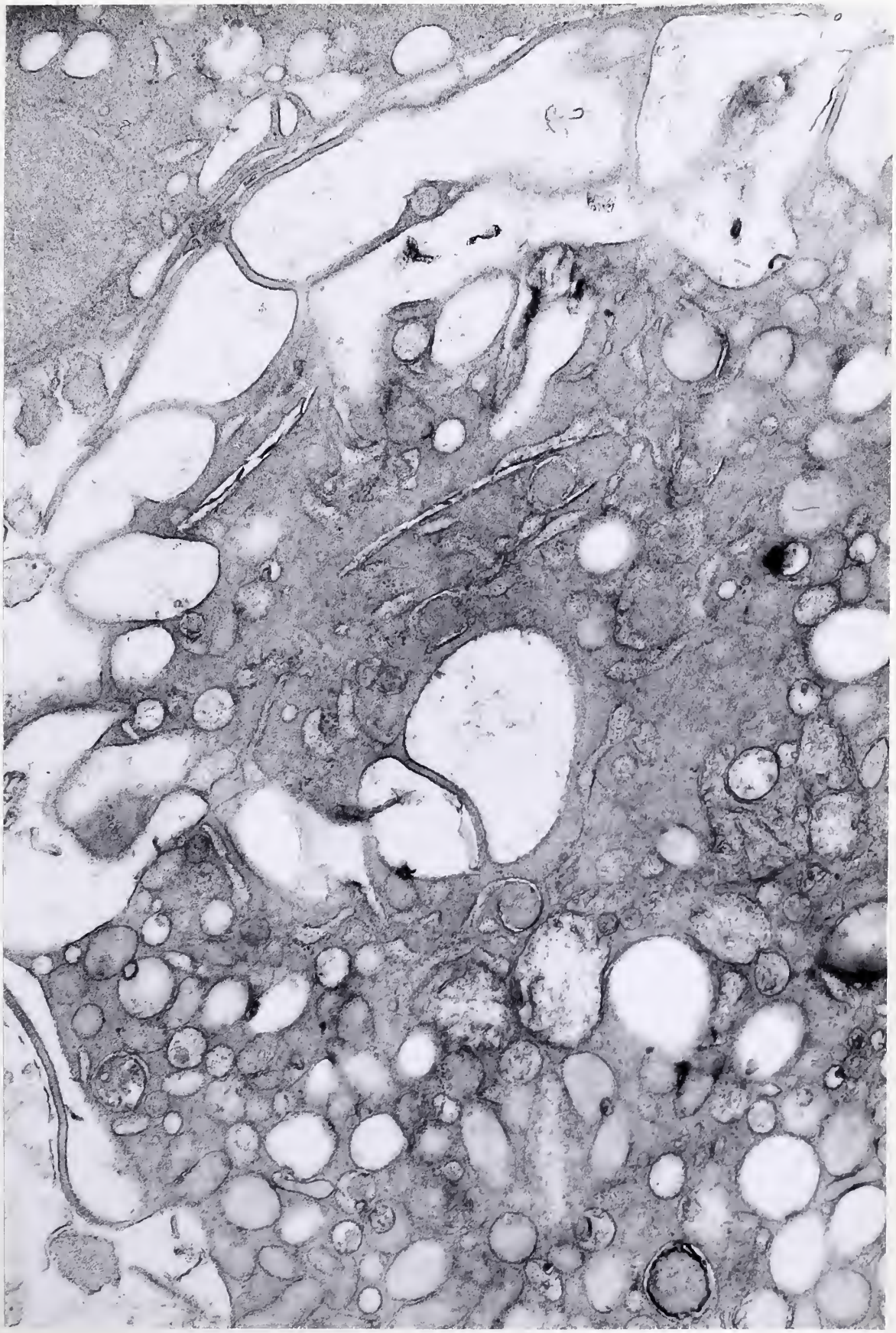


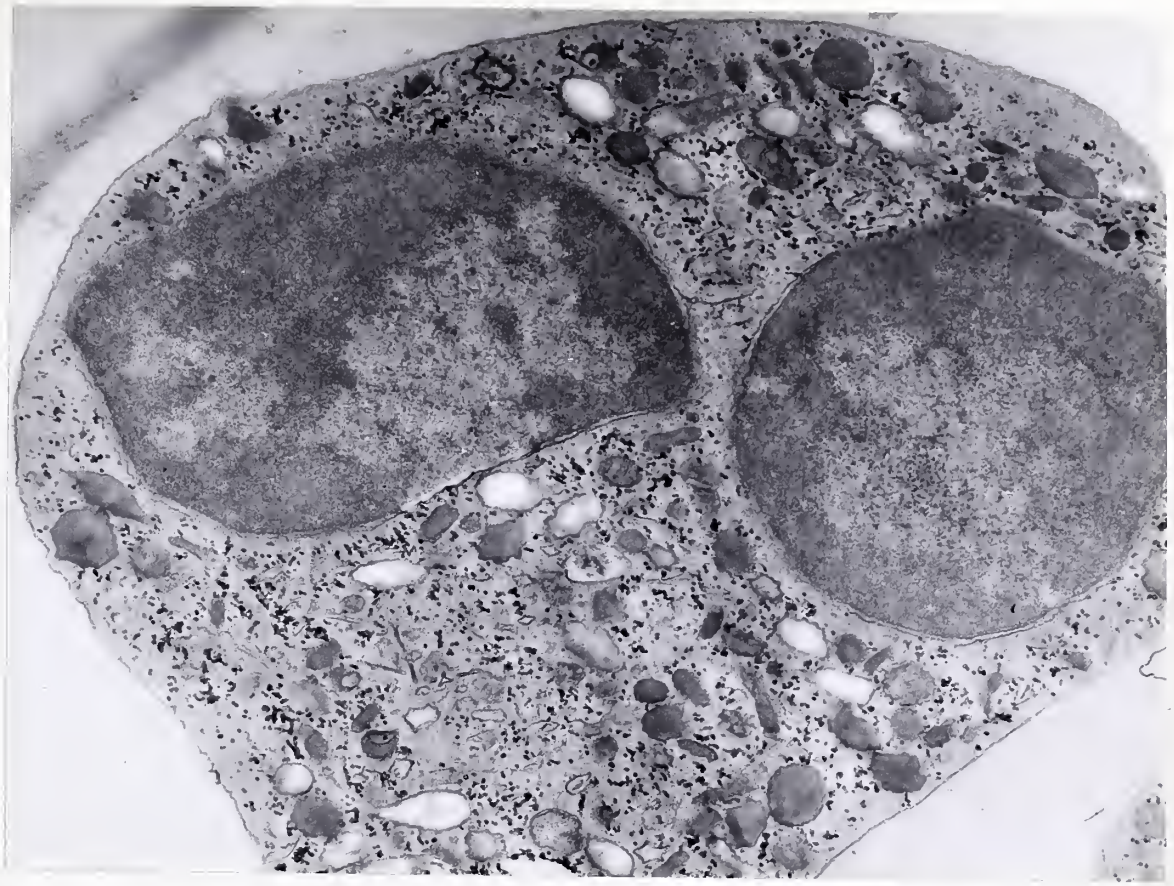
Figure 26 (above)

Control human PMN leucocyte. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X33,000.

Figure 27 (below)

Etiocholanolone-treated human PMN leucocyte. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X33,000.

Although not definitely implicated in EP release when incubated with etiocholanolone, the etio-treated PMN leucocyte (Figures 25 and 27) is different in many ways from the control PMN leucocyte (Figure 26). Cytoplasmic veils, absent in control cells, are common in etio-treated cells. The presence of many digestive vacuoles probably accounts for the larger size of the etio-treated cell. The chromatin material is less condensed in the etio-treated PMN leucocyte, and juxtanuclear whorls of membrane are more common than in the control PMN leucocyte. Deposits of endogenous cytoplasmic glycogen are distinctly less abundant in the etio-treated cells. Elements of endoplasmic reticulum are more common in the etio-treated cell. In general, fewer granules (lysosomes) are found in etio-treated PMN leucocytes, although this observation did not hold for all cells examined (e.g. Figure 27). The ground hyaloplasm of the etio-treated cell was less dense than that of the control cell.



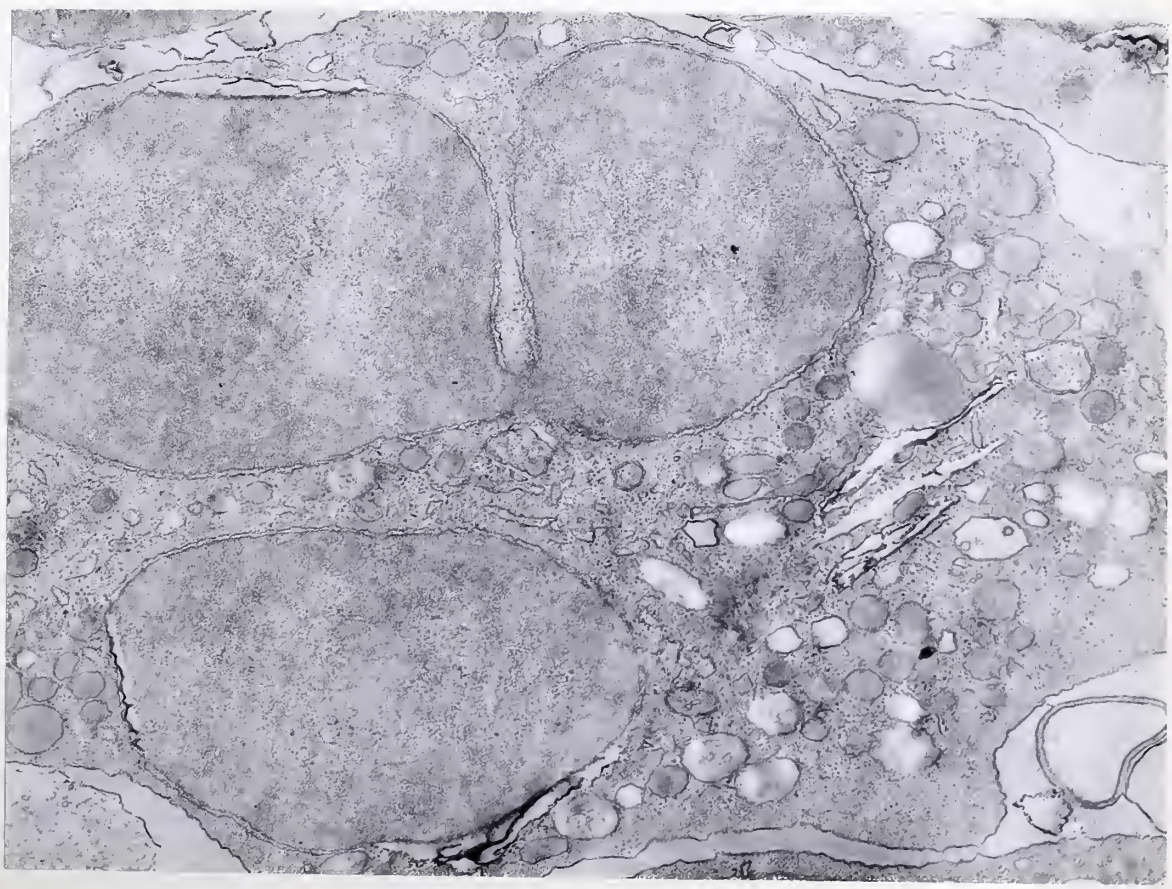


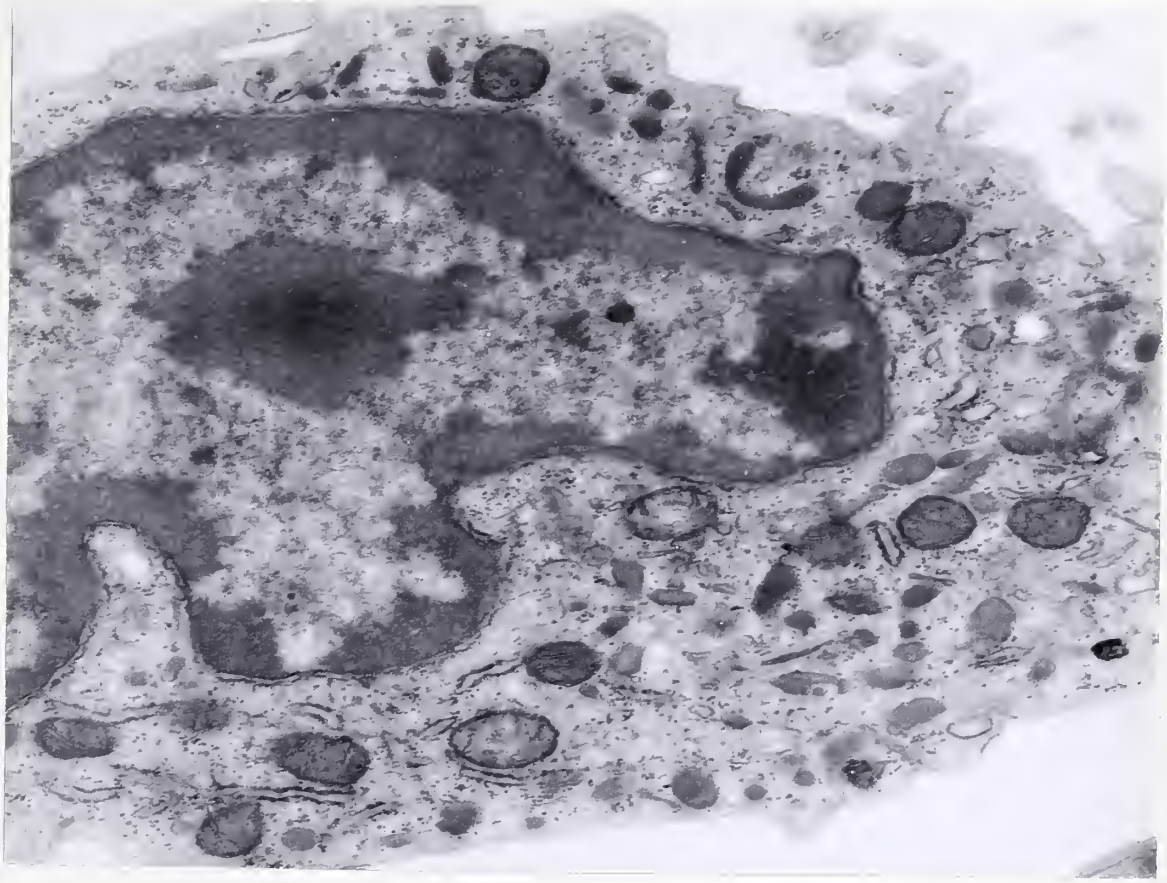
Figure 28

Control human monocyte. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X38,000.

Figure 29

Human monocyte incubated with staphylococci. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X29,000.

In whole blood incubated in the ratio 10 bacteria per leucocyte, few bacteria were phagocytosed by monocytes. Only one digestive vacuole is seen in the micrograph of a staph-treated monocyte. The chromatin material of the staph-treated cell is less condensed than that of the control, although its distribution is similar. Whorls of perinuclear membrane were seen with similar frequency in both populations. Although lysosomes seemed less prevalent in staph-treated cells, the numbers or distribution of the other cytoplasmic structures was similar.



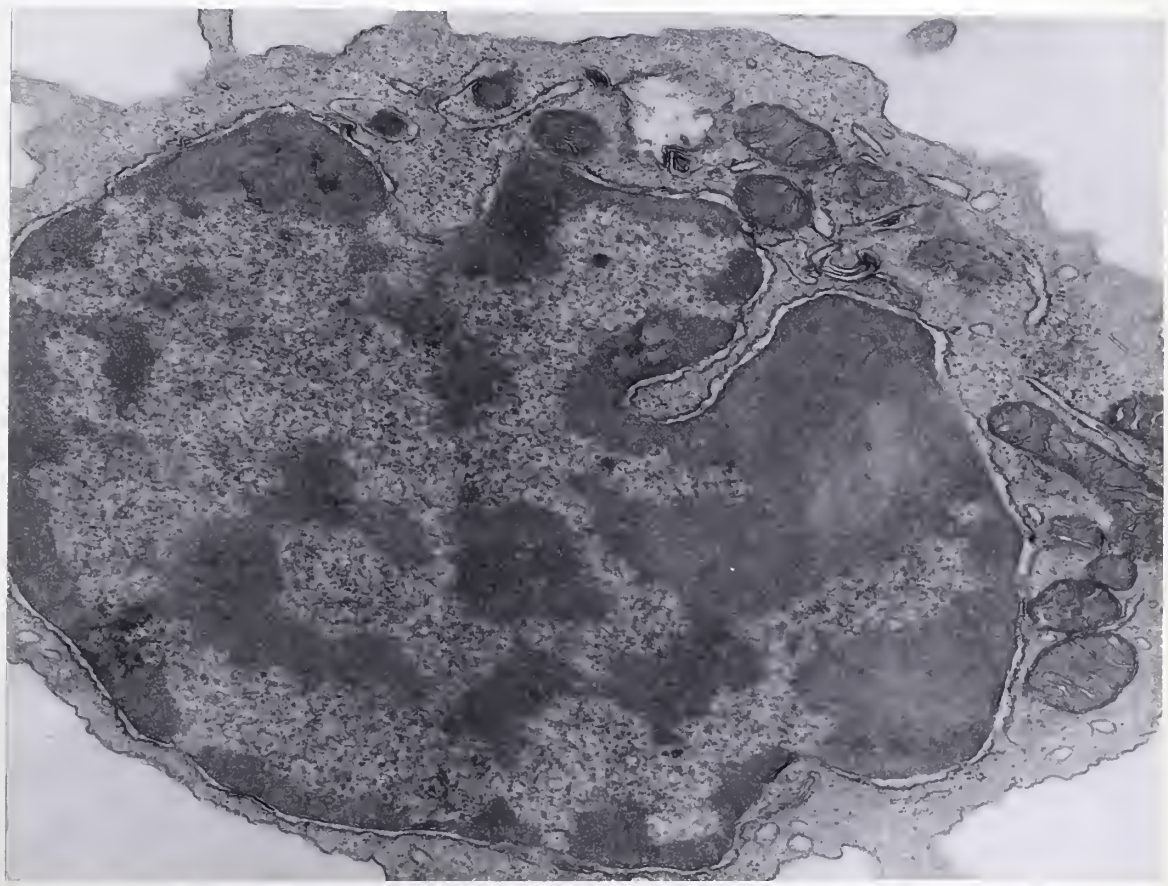


Figure 30

Human PMN leucocyte incubated with staphylococci. Five large digestive vacuoles, two of which contain intact staphylococci, are seen in the lower right of this micrograph. A description of the differences between this cell and controls can be seen on the following page. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X42,000.

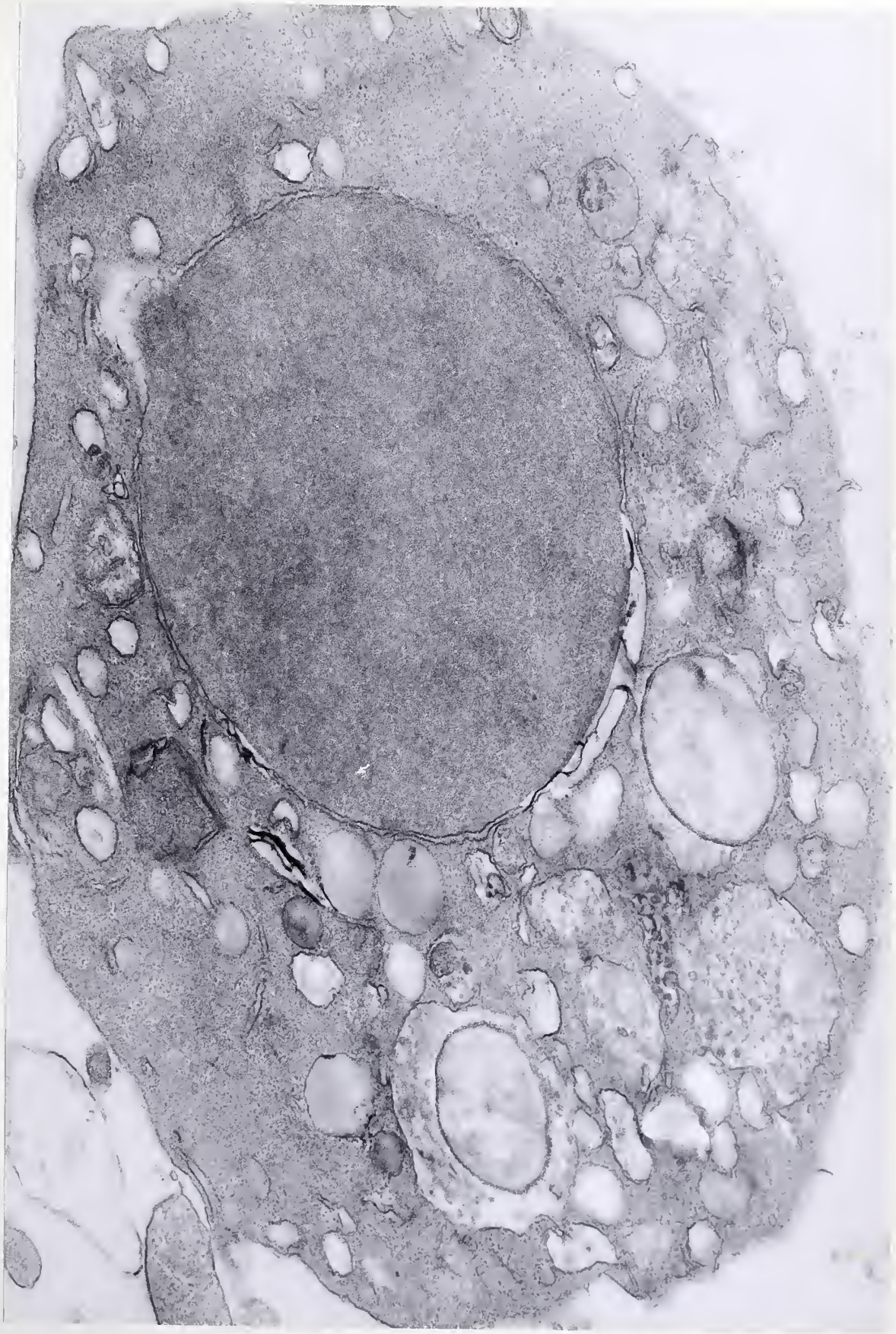


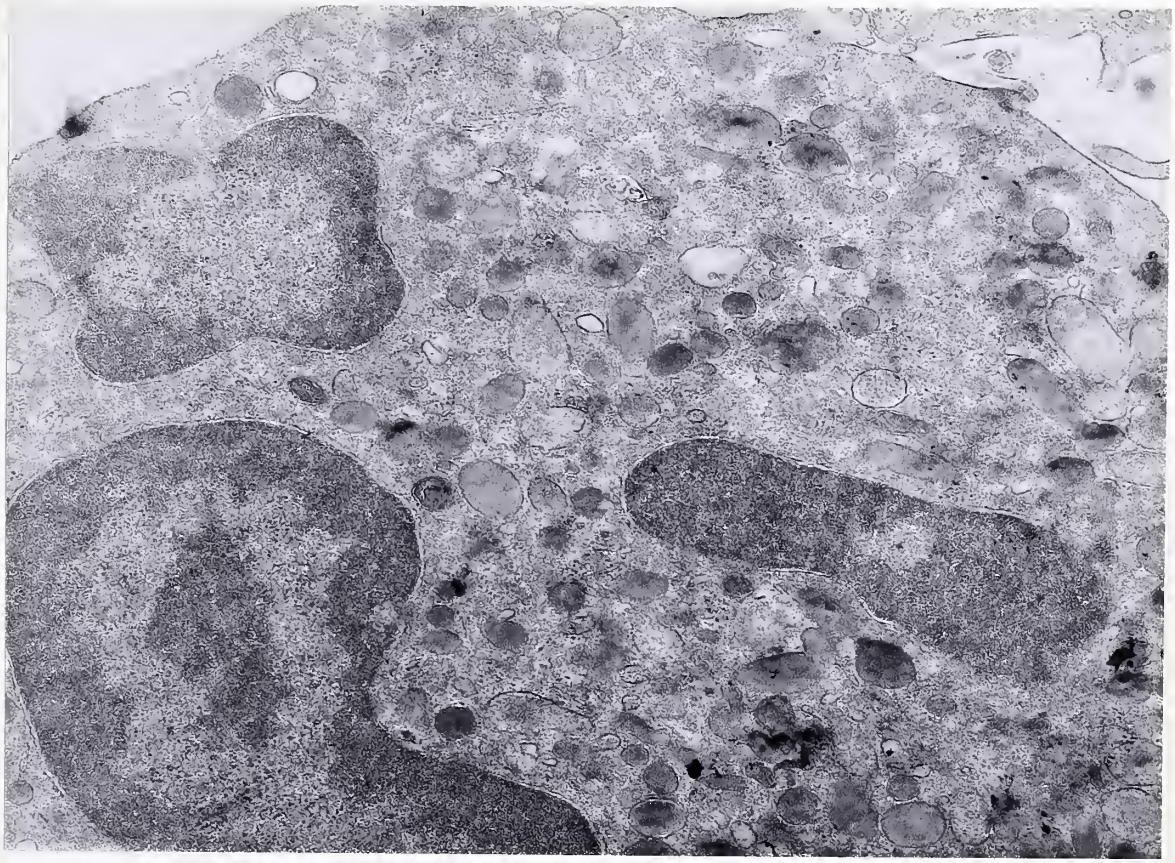
Figure 31 (above)

Control human PMN leucocyte. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X29,000.

Figure 32 (below)

Human PMN leucocyte incubated with staphylococci. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X27,000.

PMN leucocytes incubated with staphylococci (e.g. Figures 30 and 32) abound with digestive vacuoles containing phagocytosed staphylococci; the control cells (e.g. Figure 31) have none. The surface of the staph-treated cell is more irregular because of the presence of more pseudopodia; cytoplasmic veils are rare in both populations. Elements of endoplasmic reticulum and Golgi apparatus are more frequently seen in staph-treated cells; deposits of cytoplasmic glycogen are significantly less prevalent than in controls. In general, staph-treated cells contain fewer granules (lysosomes), although this is not evident in every cell. PMN leucocytes incubated with staphylococci do not stain as heavily in lead citrate as controls, and slight overstaining like that seen in Figure 31 does not occur as readily. No difference in nuclei or nuclear membranes between the two populations is evident.



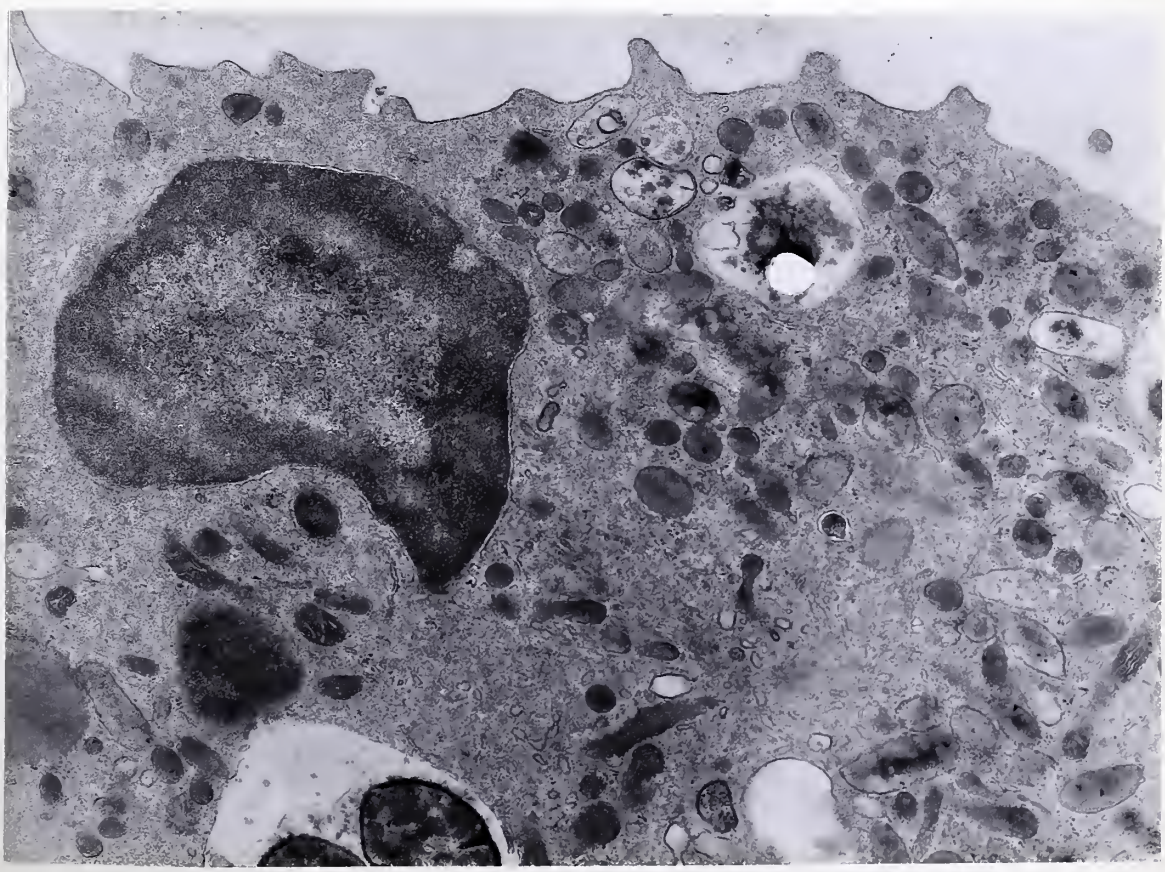


Figure 33

Human PMN leucocyte incubated with staphylococci (PTA stain). In the left center of this micrograph, a centriole with microtubules radiating from it is seen in cross-section. The differences between PMN leucocytes incubated with staphylococci and controls will be discussed on the following page. Fixation in glutaraldehyde. PTA stain. X46,000.

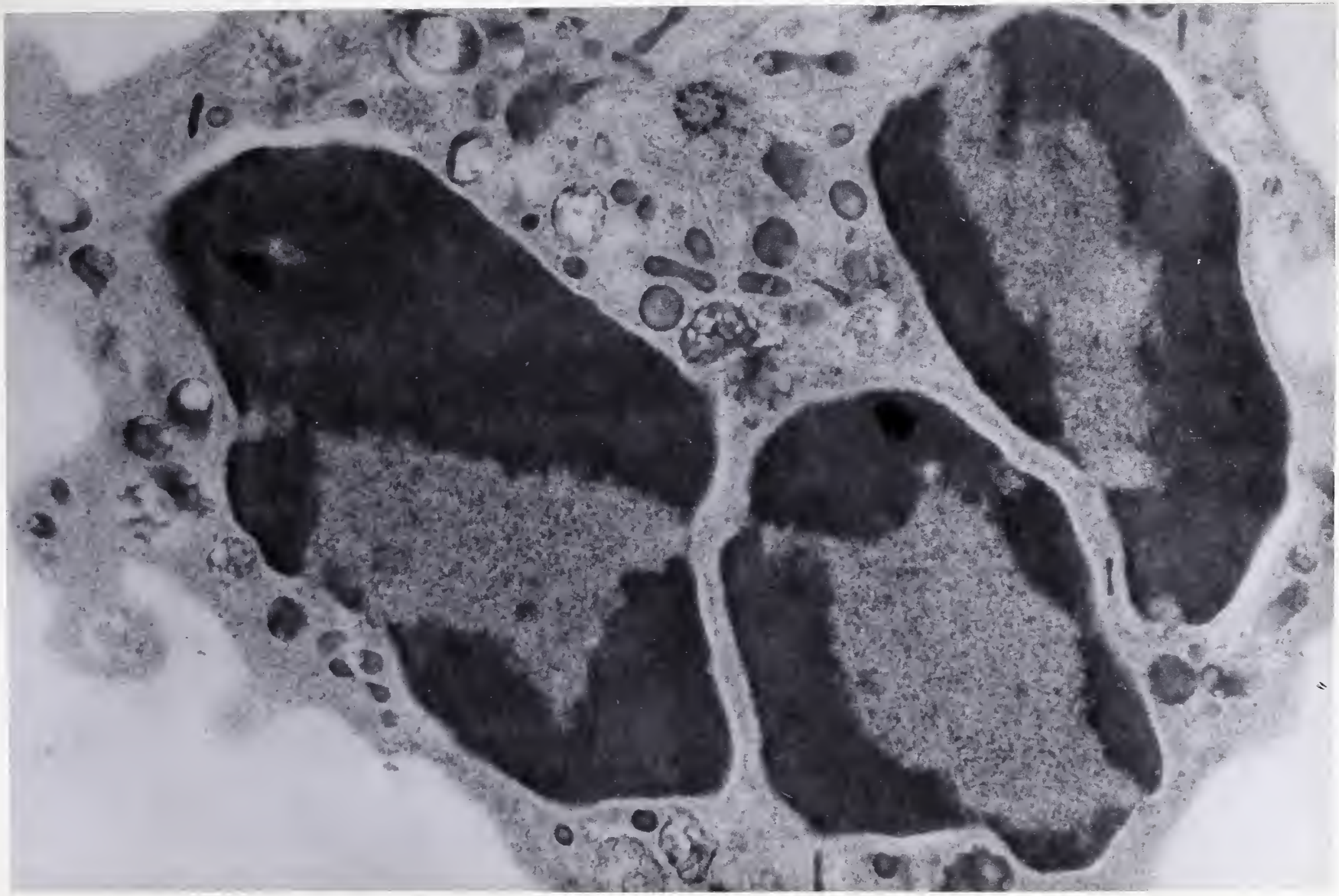


Figure 34 (above)

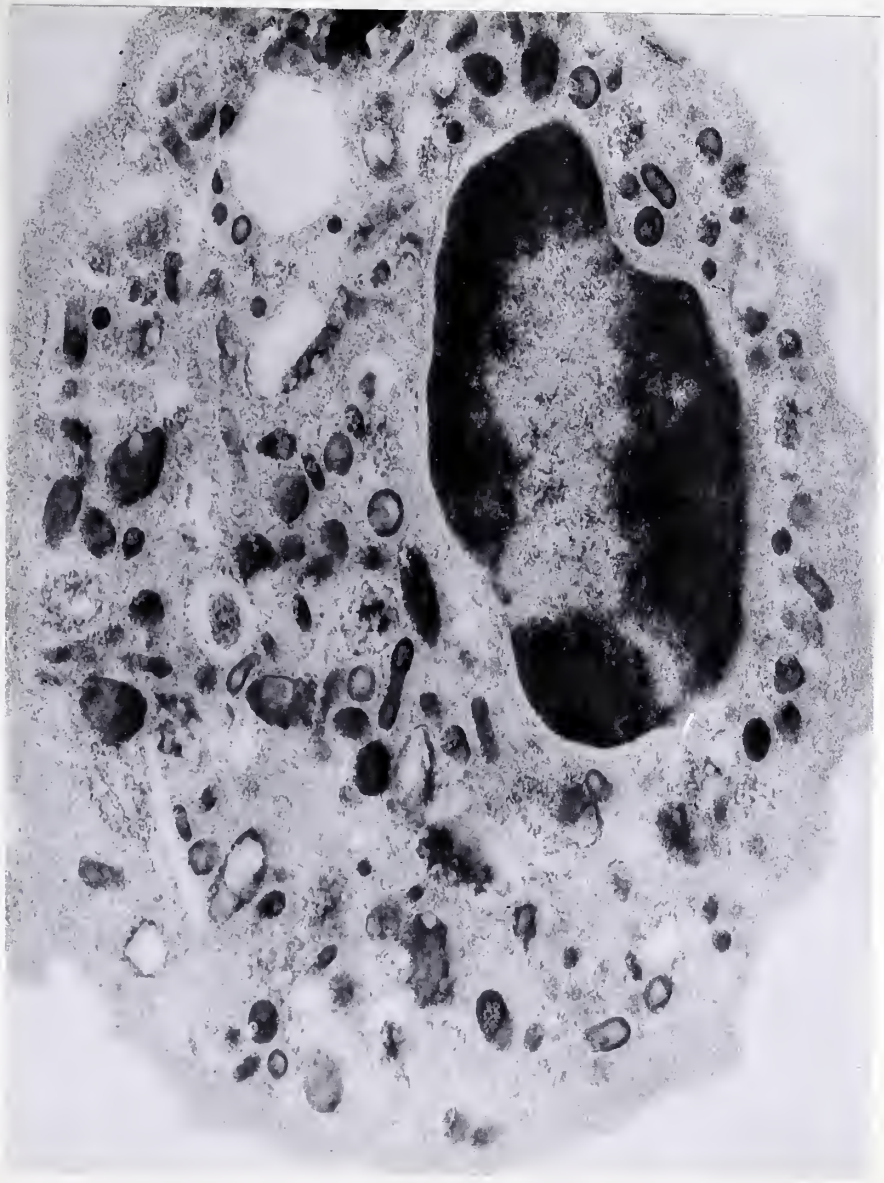
Control human PMN leucocyte (PTA stain). Fixation in glutaraldehyde. PTA stain. X31,000.

Figure 35 (below)

Human PMN leucocyte incubated with staphylococci (PTA stain). Fixation in glutaraldehyde. PTA stain. X31,000.

The morphology of control PMN leucocytes is identical to that described earlier for normal PMN leucocytes in PTA stain. The dense nuclear chromatin is clumped centrally. Only a small fringe of chromatin is closely apposed to the nuclear envelope throughout the entire circumference not adjacent to the central clump. The nucleoplasm is relatively lucent, and no structures resembling nuclear pores are seen. Centrioles and microtubules are seen rarely. The ground hyaloplasm is relatively dense.

In staph-treated PMN leucocytes, the nuclear morphology is quite different, resembling that seen in normal monocytes and eosinophils. The chromatin is not as clumped, and is arranged peripherally, adjacent to the nuclear envelope. In areas where chromatin is not apposed to the nuclear envelope, the juxtanuclear cytoplasmic density is often increased and the cistern of the nuclear envelope appears denser. These areas, absent in controls, are thought to represent nuclear pores. Centrioles and microtubules are seen commonly. The ground hyaloplasm is relatively lucent. In addition, there appear to be fewer lysosomes in staph-treated PMN leucocytes.



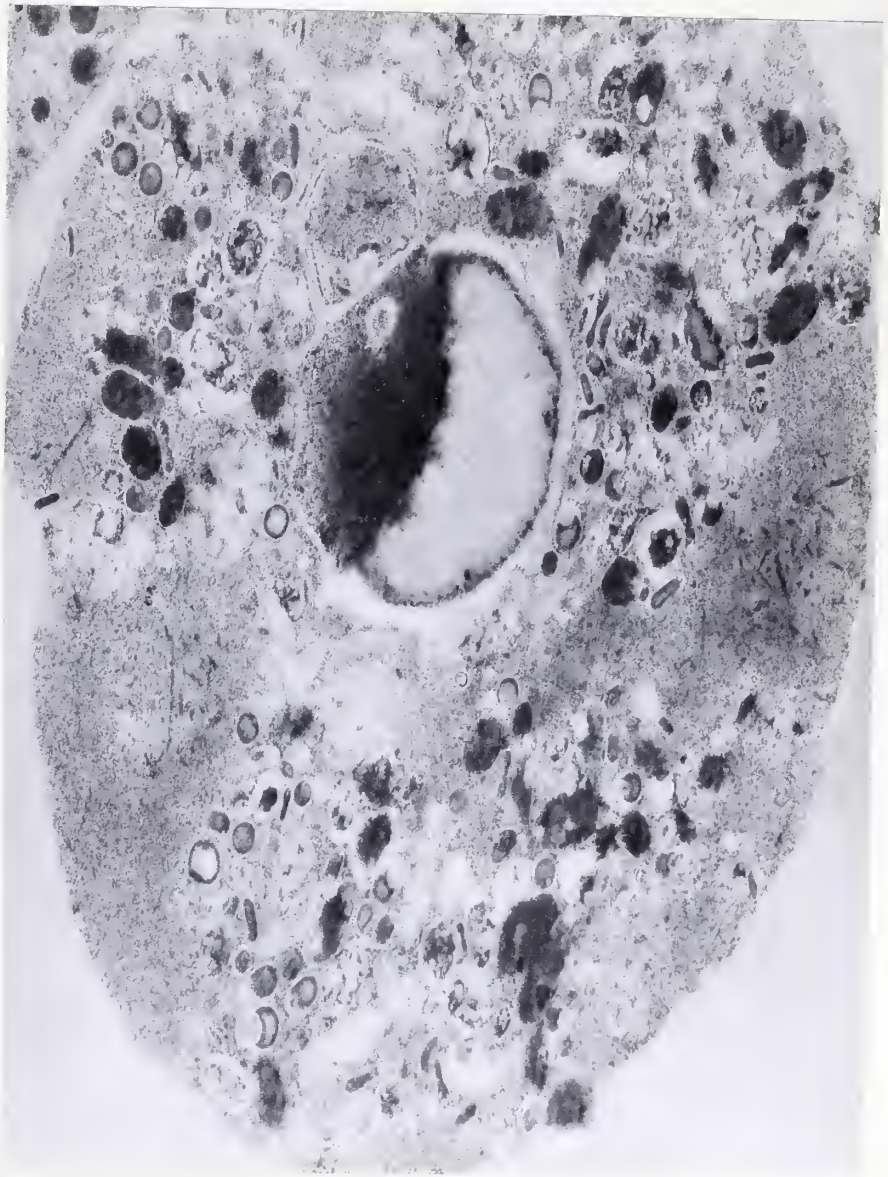


Figure 36 (top left)

Human PMN leucocyte incubated with staphylococci (Stained with PTA). A few PMN leucocytes in each preparation treated with staphylococci display morphologies inconsistent with that described for staph-treated PMN leucocytes. These atypical cells account for less than 5% of the total number seen. The nuclear chromatin distribution and ground hyaloplasm density of this cell resemble that of a control cell, although it is from a population incubated with staphylococci. Fixation in glutaraldehyde. PTA stain. x20,000.

Figure 37 (top right)

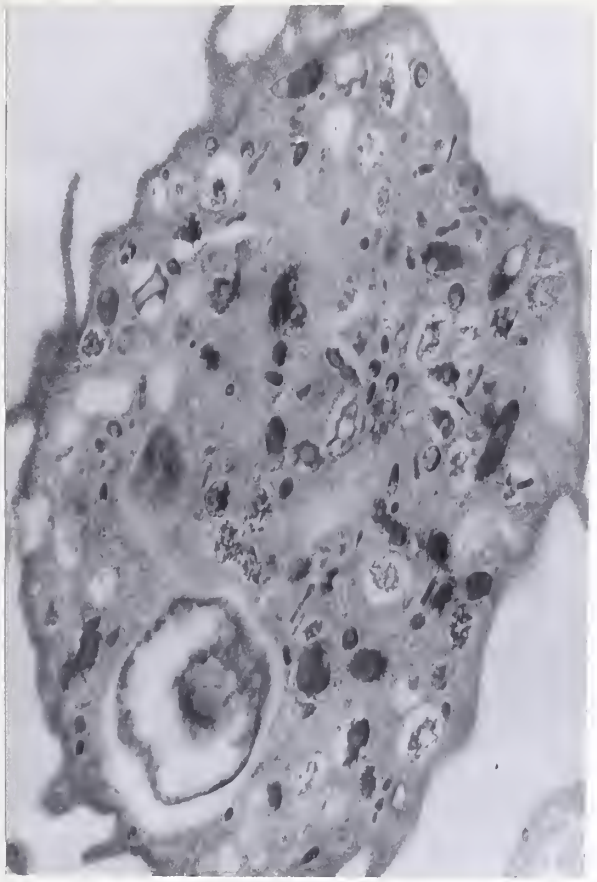
Control human PMN leucocyte (stained with PTA). About 5% of PMN leucocytes from control preparations possess morphologies different from the typical control cell morphology. The distribution of chromatin density and density of ground substance in this cell appear intermediate between that found in typical control and typical staph-treated PMN leucocytes. Fixation in glutaraldehyde. PTA stain.X20,000.

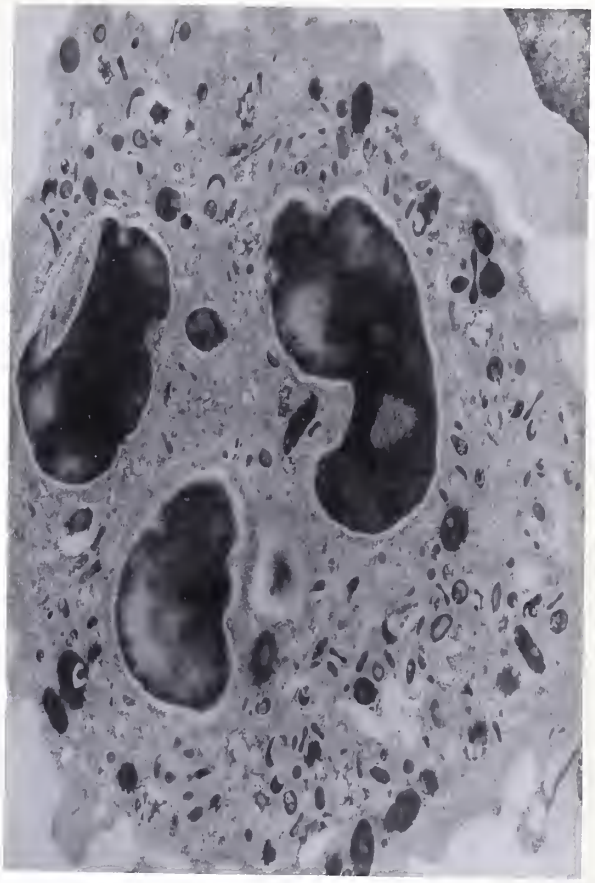
Figure 38 (lower left)

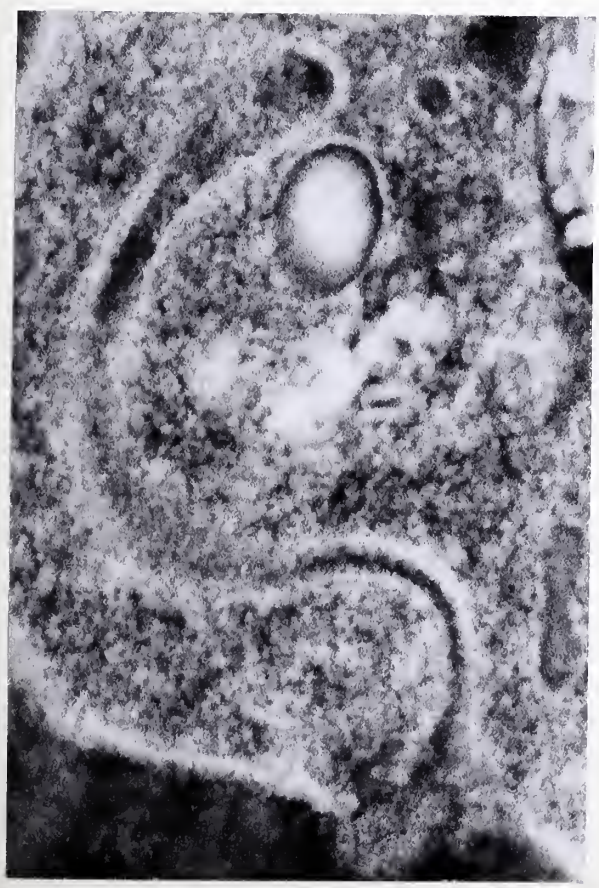
Human PMN leucocyte incubated with actinomycin (stained with PTA). A high magnification view of a strand of nucleus connecting two lobes of a PMN nucleus, one of which is at the bottom of the micrograph ; the other lobe is out of the plane of section. Fixation in glutaraldehyde.PTA stain. X109,000.

Figure 39 (lower right)

Human PMN leucocyte incubated with staphylococci (stained with PTA). A longitudinal section through a centriole is seen in the lower part of this micrography. Microtubules radiating from it can also be seen. Fixation in glutaraldehyde. PTA stain. X109,000.







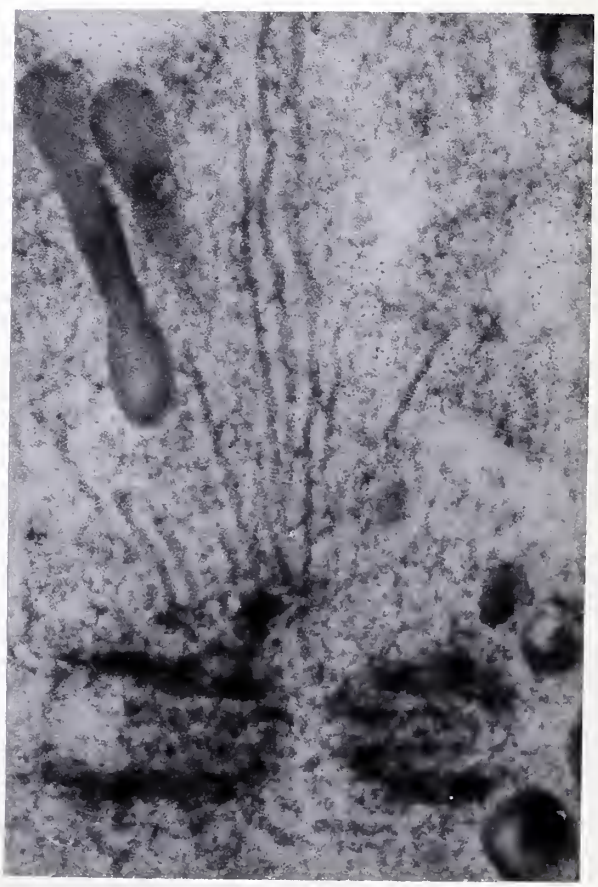


Figure 40

Human monocyte incubated with actinomycin-D. This cell does not appear significantly different from normal monocytes. In other monocytes treated with actinomycin, threadlike densities in the nucleus similar to those seen in Figures 41-43 can be seen. Although actinomycin-D has been shown to have profound effects on RNA biosynthesis and eventual protein synthesis of cells, the fine structural correlates of these biochemical changes are minimal in human blood cells. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X36,000.

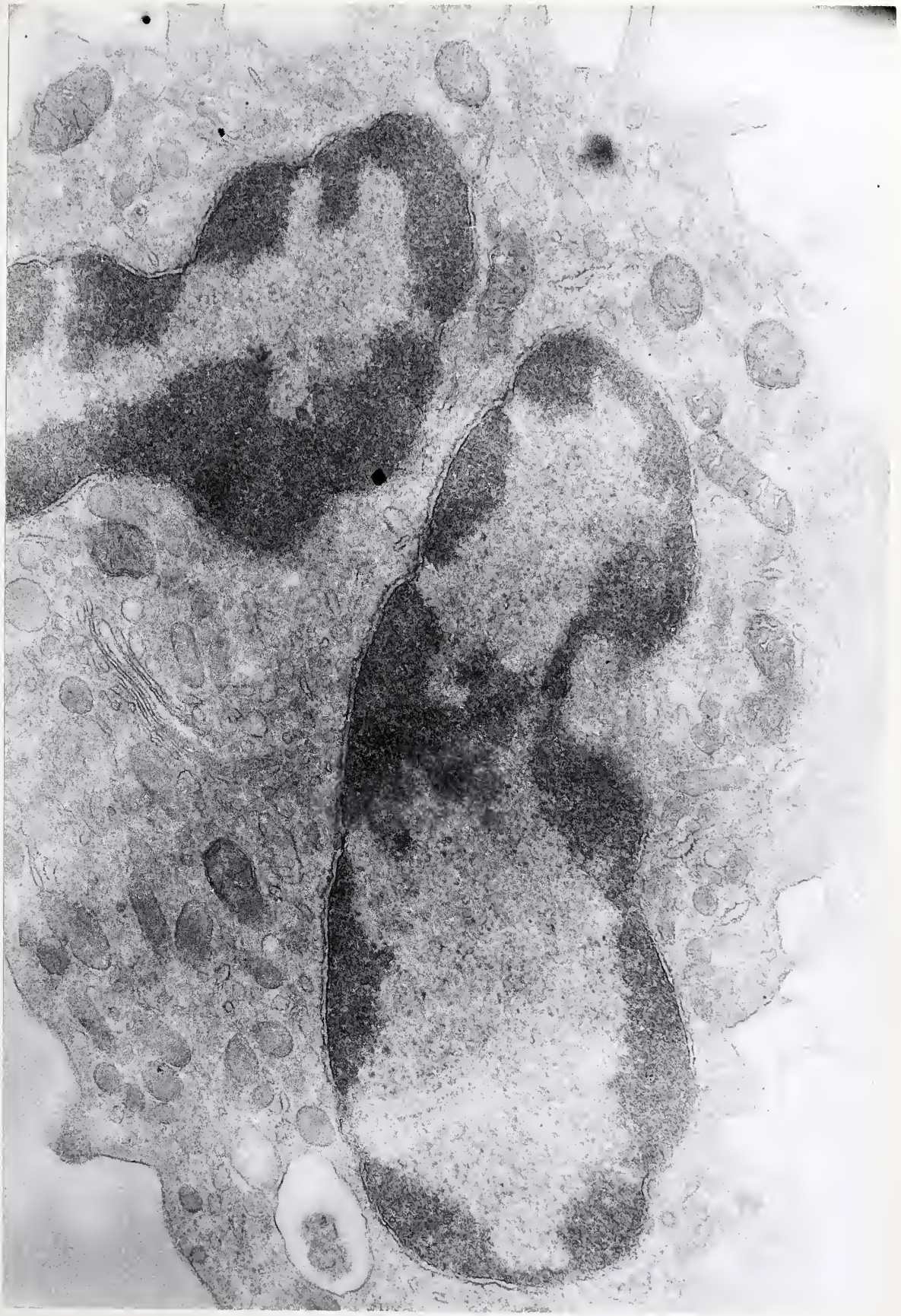
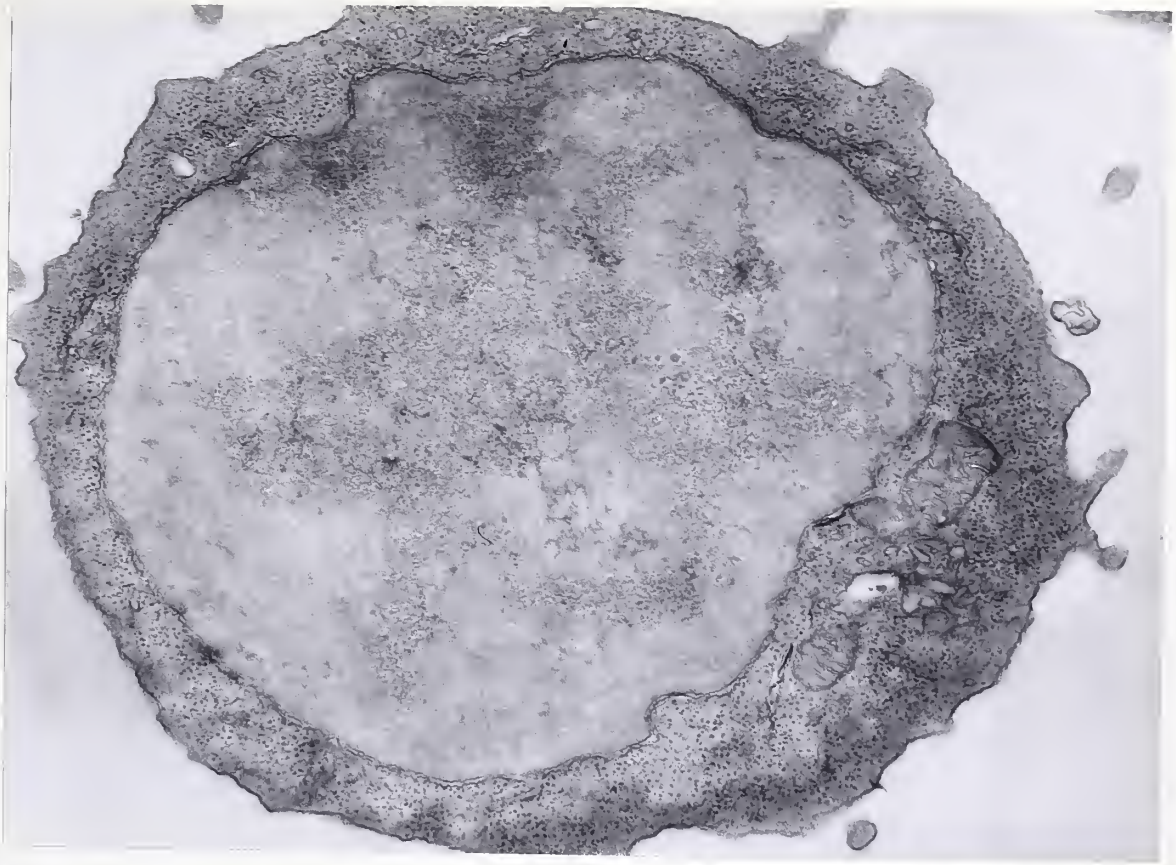


Figure 41

Human lymphocyte incubated with actinomycin-D. The morphology of this cell is different from normal monocytes in only one detectable respect. Within the nucleus, skeins of fine, threadlike densities can be seen. This finding is extremely common in lymphocytes treated with actinomycin, but rare in normal lymphocytes. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X34,000.

Figure 42

Human eosinophil incubated with actinomycin-D. This cell is similar to normal eosinophils except for the appearance of skeins of threadlike density which are found within the nucleus. This change is found commonly in human leucocytes treated with actinomycin. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X34,000.



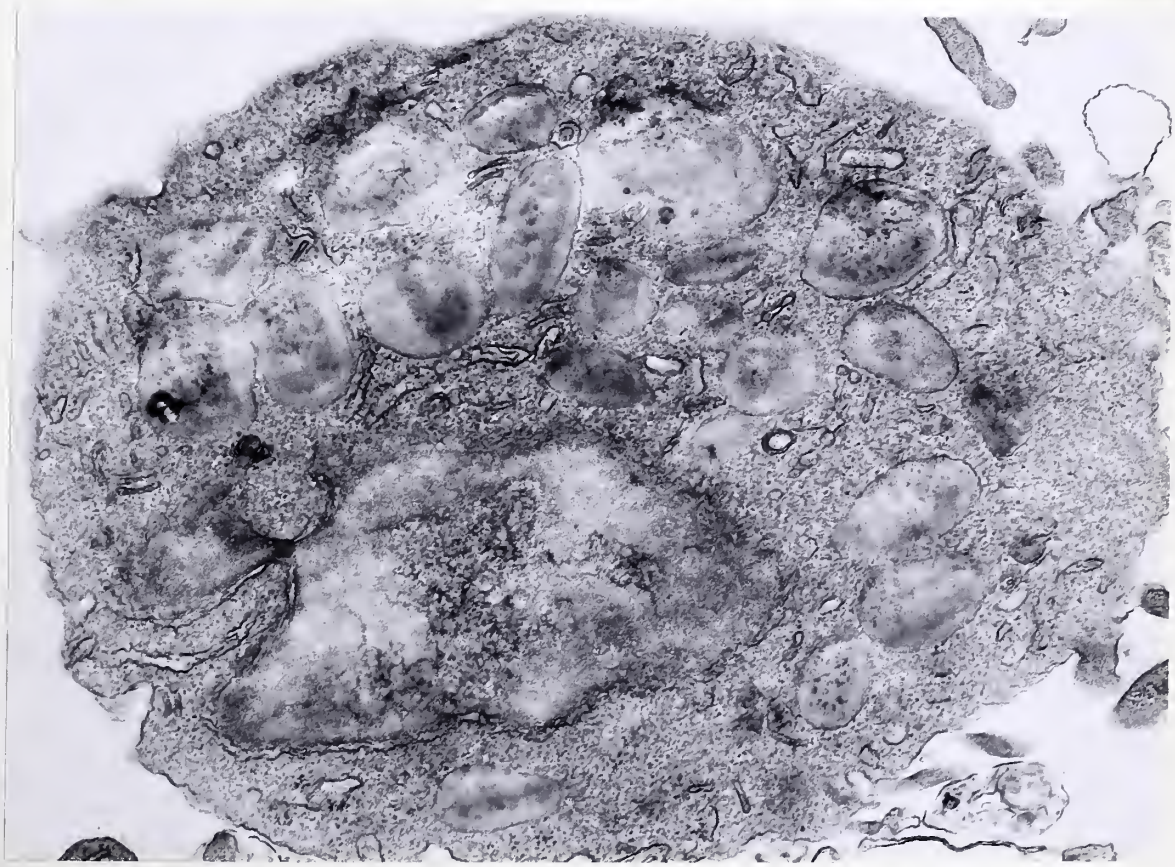


Figure 43

Human PMN leucocyte incubated with actinomycin-D. Only three differences between this cell and normal PMN leucocytes can be distinguished. The dense, threadlike deposits within the nucleus are seen only rarely in normal PMN leucocytes. Normal cells do not demonstrate as great an affinity for lead citrate as do cells treated with actinomycin. Finally, there are significantly fewer lysosomes (granules) in the actinomycin-treated PMN leucocyte. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X42,000.

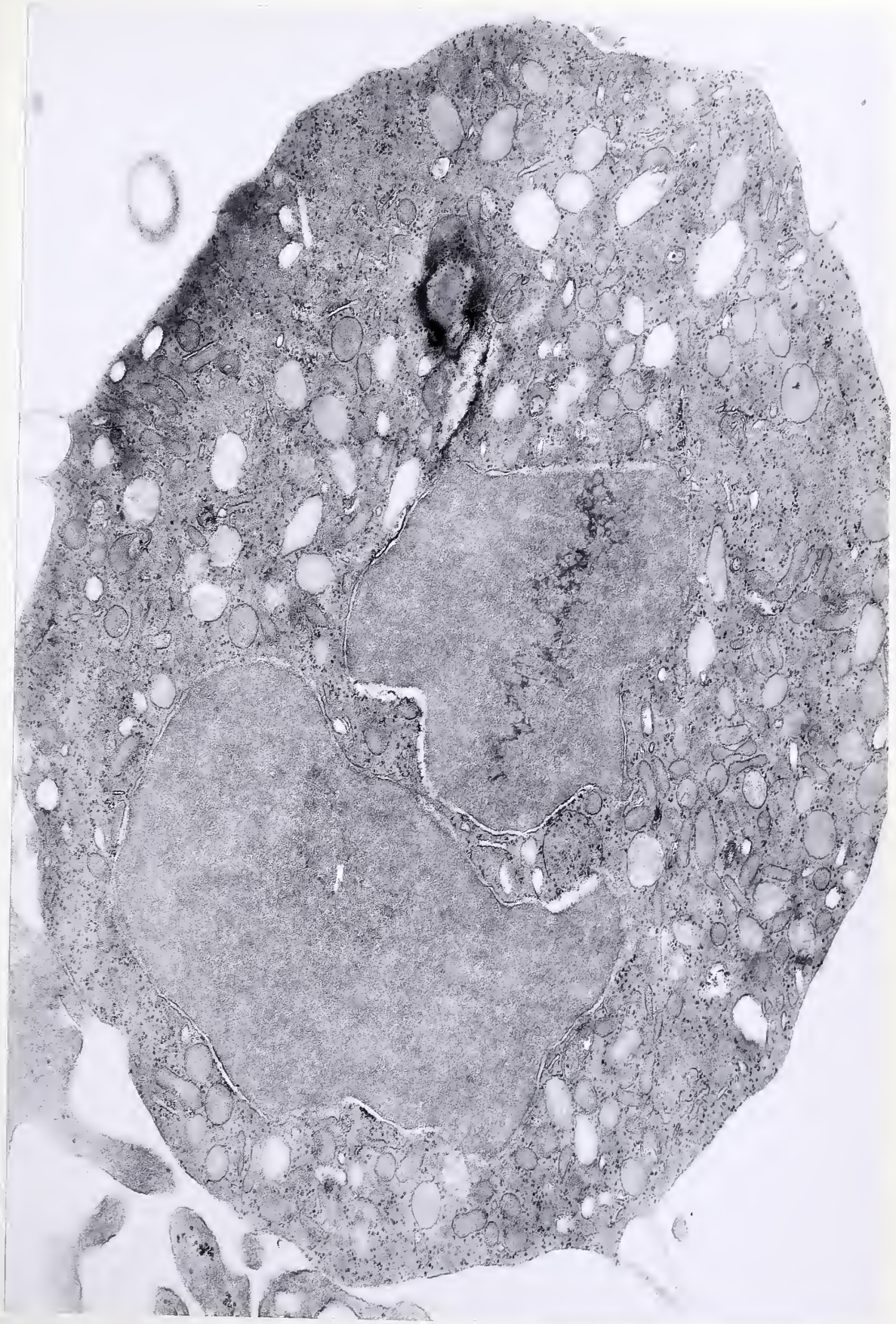


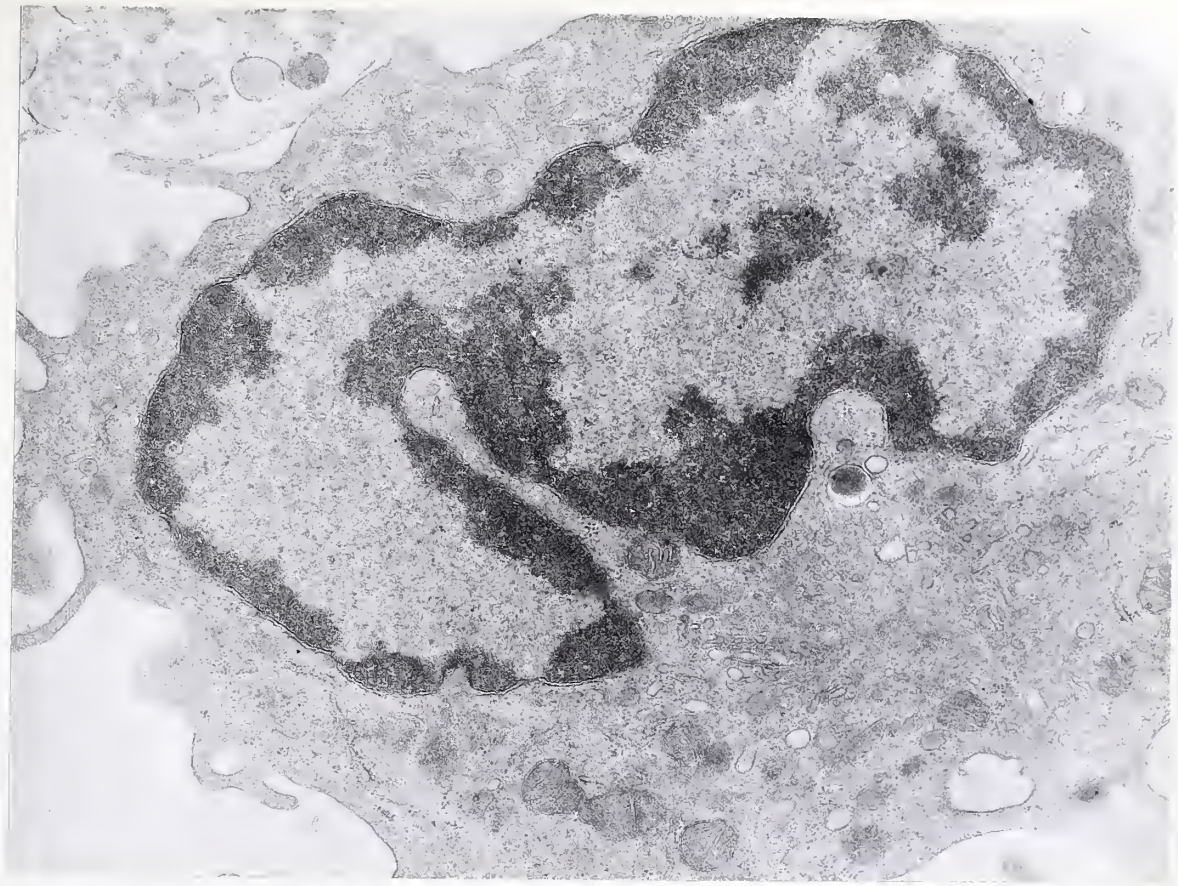
Figure 44 (above

Human monocyte incubated with actinomycin-D (actinomycin control). Fixation in glutaraldephyde and osmium tetroxide. Lead citrate stain. X24,000.

Figure 45 (below)

Human monocyte incubated with staphylococci and actinomycin-D. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X23,000.

When these two cells are compared, it can be seen that the chromatin material of the staph-actinomycin treated cell is less condensed than that of the actinomycin control monocyte. Elements of endoplasmic reticulum and Golgi apparatus are more commonly encountered in the staph-actinomycin treated cell, and the cell surface is more irregular than in the actinomycin control cell. No difference in the population of lysosomes or mitochondria was found.



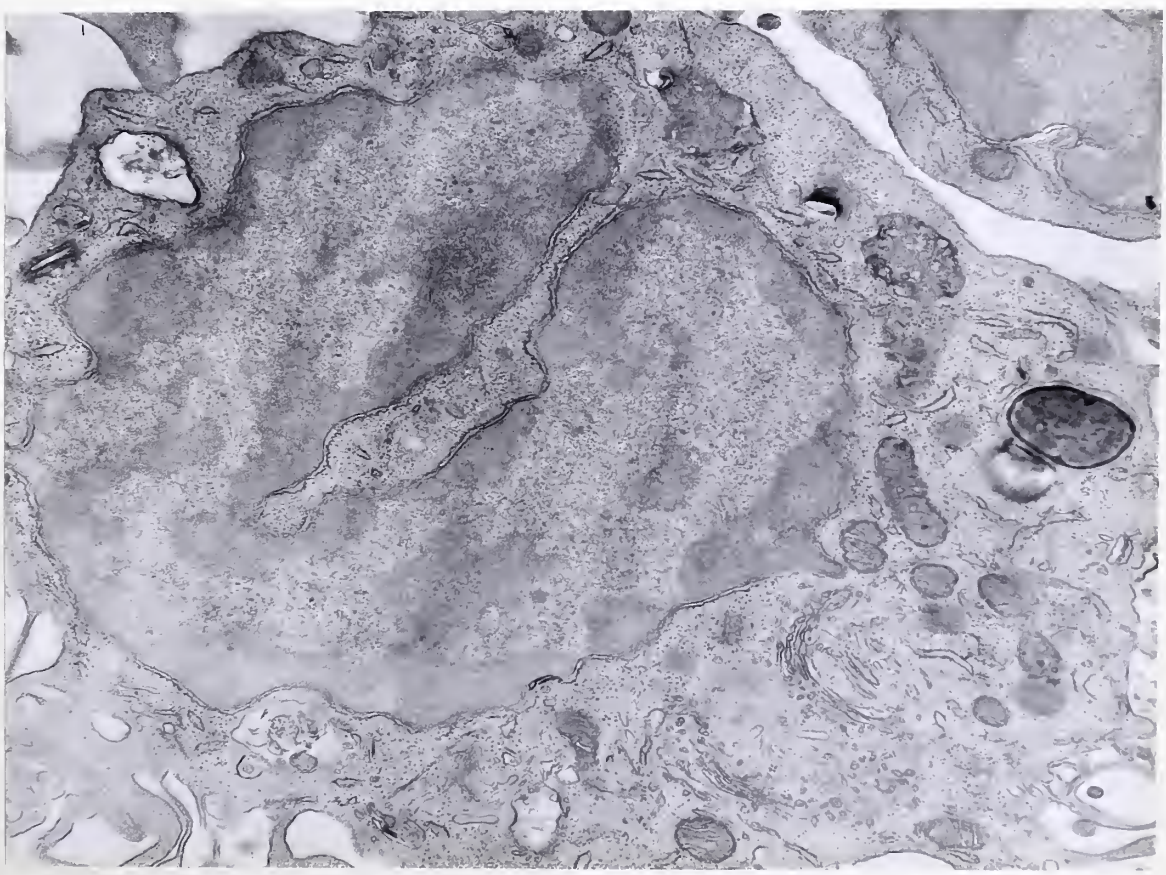


Figure 46

Human PMN leucocyte incubated with staphylococci and actinomycin-D. A description of the differences between this cell and actinomycin control cells appears on the next page. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X38,000.

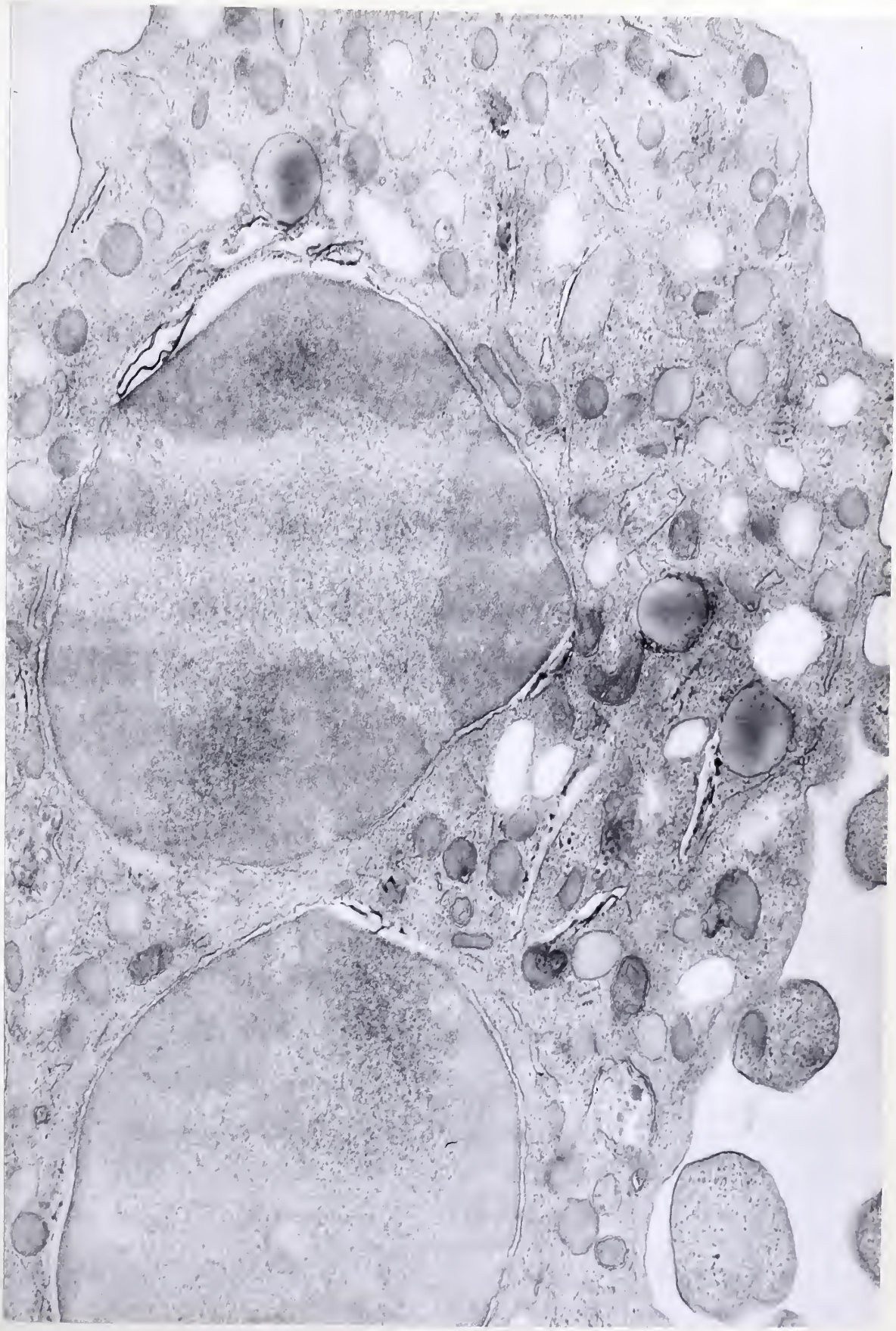


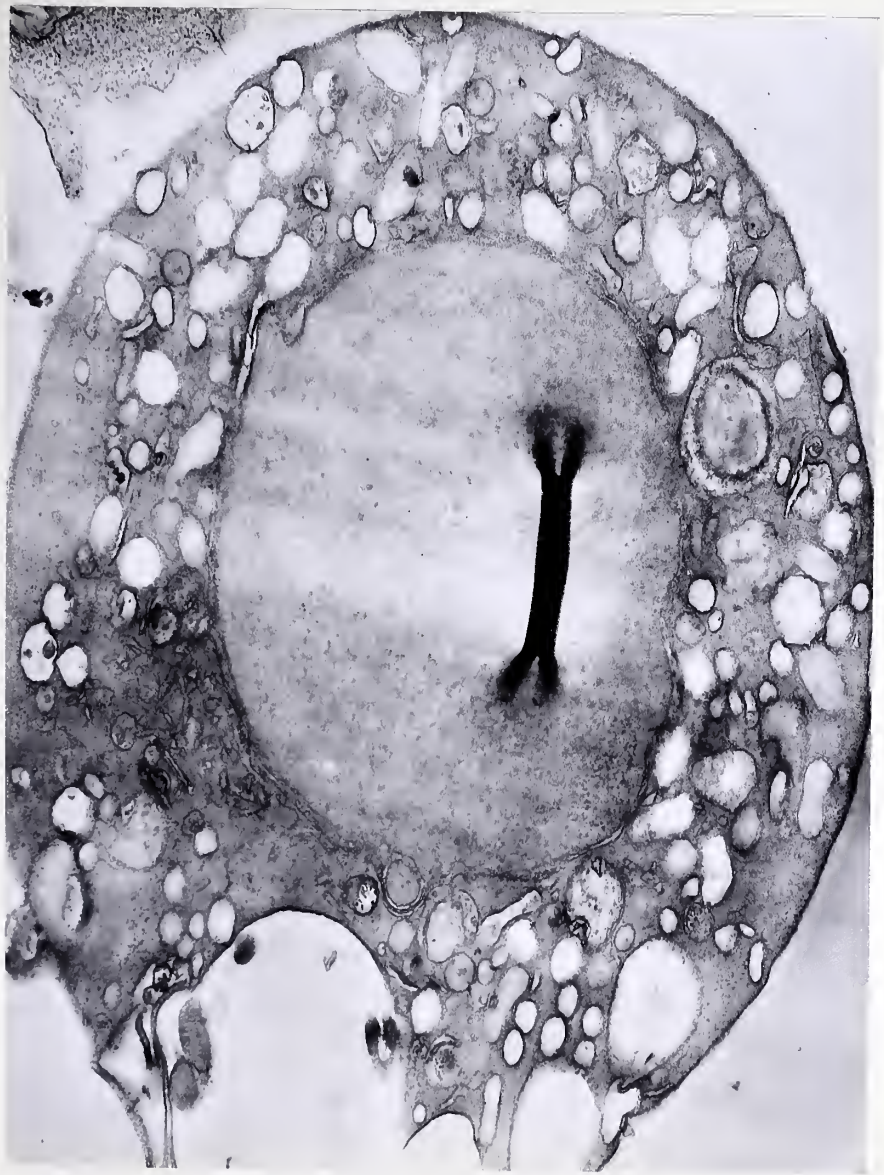
Figure 47 (above)

Human PMN leucocyte incubated with actinomycin-D (actinomycin control). A fold in the section can be seen traversing the upper part of this cell. Skeins of threadlike density appear in the nucleus. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X35,000.

Figure 48 (below)

Human PMN leucocyte incubated with staphylococci and actinomycin-D. A section fold can be seen. Large digestive vacuoles occupy much volume in the right of this micrograph. Skeins of threadlike nuclear density are seen rarely in these cells. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X26,000.

The larger size of the staph-actinomycin treated cell can probably be explained by the presence of many digestive vacuoles which are less common in actinomycin control cells. The surface of the staph-actinomycin treated cell was more irregular, elements of endoplasmic reticulum and Golgi apparatus were encountered more frequently, and less cytoplasmic glycogen was present than in actinomycin control cells. Fewer lysosomes were present in staph-actinomycin treated PMN leucocytes than in actinomycin control PMN leucocytes. Actinomycin control cells stained more heavily in lead citrate than staph-actinomycin treated cells. The differences between these two populations are similar, if not nearly identical, to the differences between control and staph-treated PMN leucocytes.



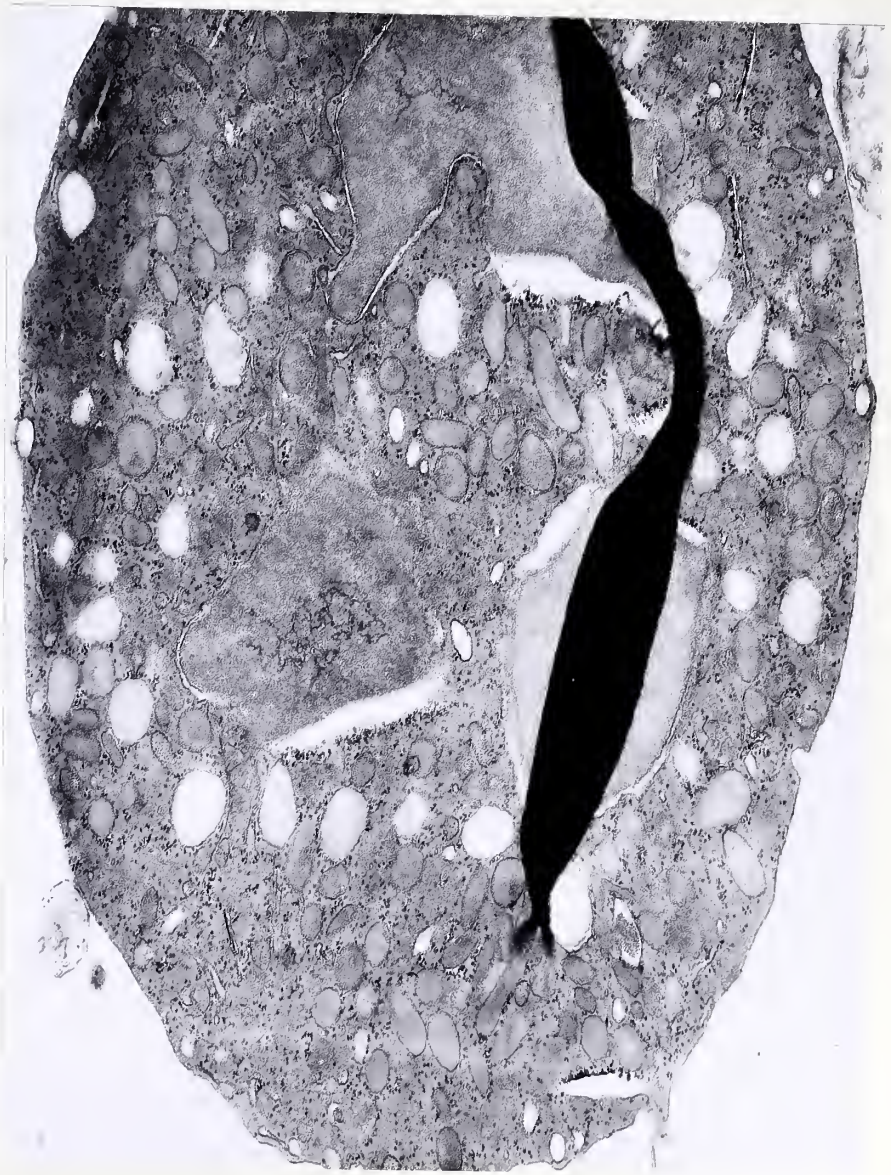


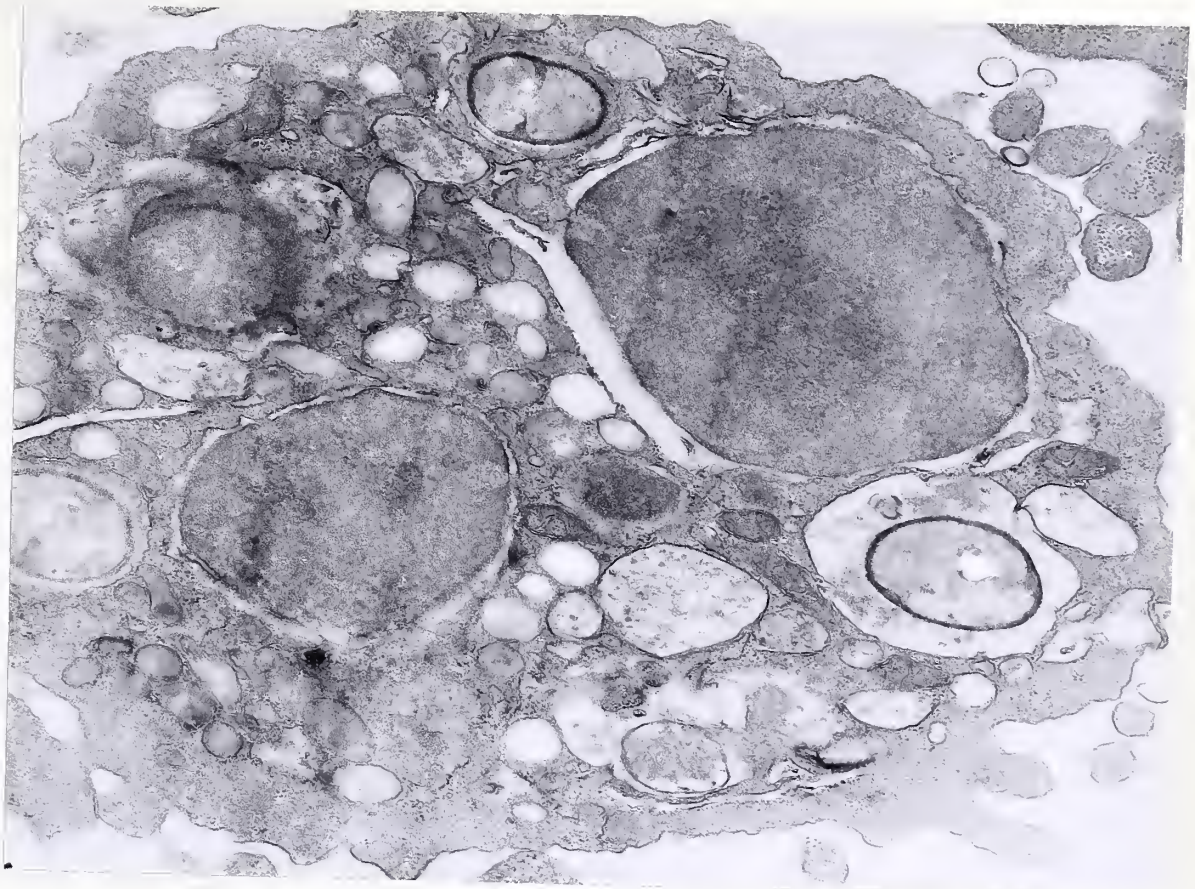
Figure 49 (above)

Human PMN leucocyte incubated with staphylococci. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X27,000.

Figure 50 (below)

Human PMN leucocyte incubated with staphylococci and actinomycin-D. Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X32,000.

Comparison of these two cells elucidates the effects produced on staph-treated cells by the subsequent addition of actinomycin-D. The only consistent difference between the two populations, of which Figures 49 and 50 are examples, is a decreased number of lysosomes (granules) in the staph-actinomycin treated group. It has been noted that actinomycin produces a decrease in the number of lysosomes in the normal cell not treated with staphylococci. Therefore, it can be stated that there is no difference between the pyrogenically active staph-treated PMN leucocytes and the pyrogenically inactive staph-actinomycin treated PMN leucocytes that cannot be attributed to an effect of actinomycin alone.



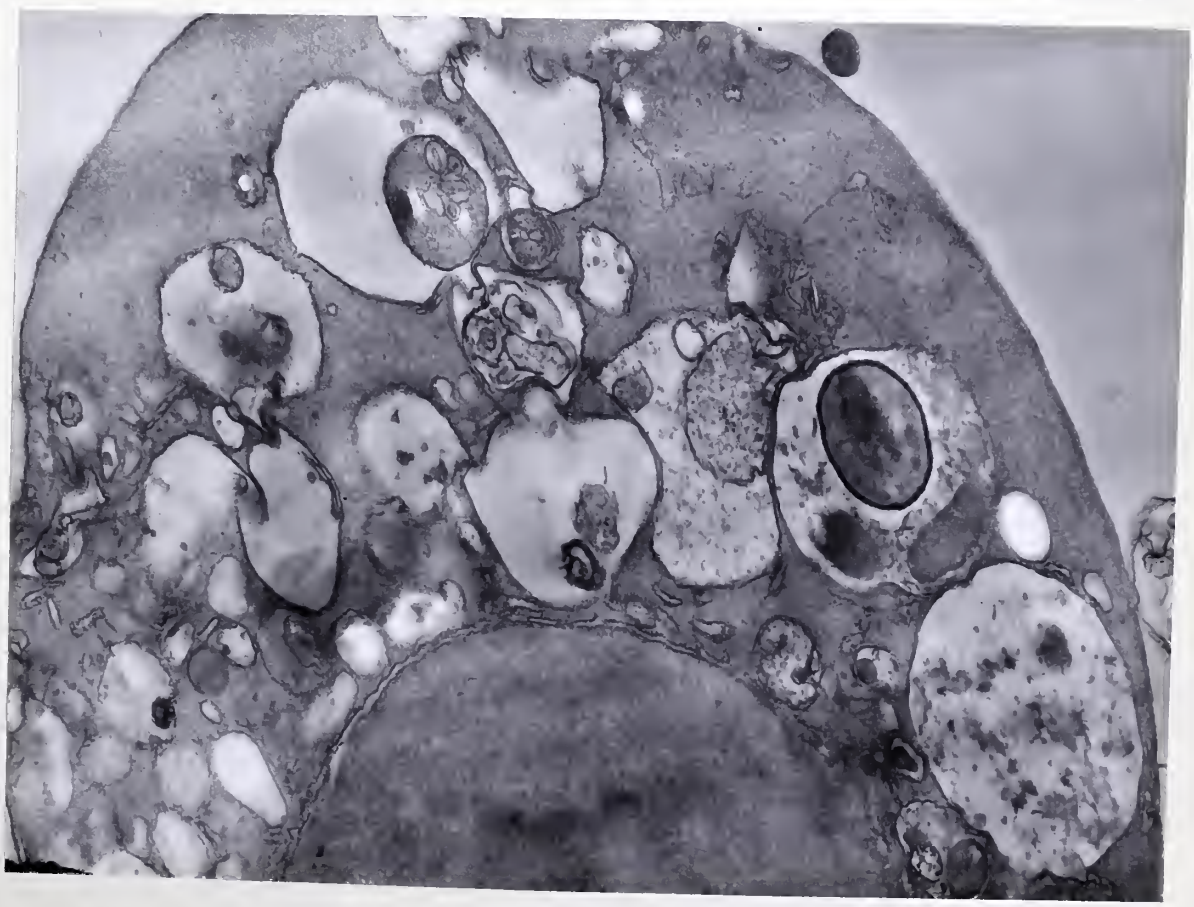


Figure 51 (top)

Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X116,000.

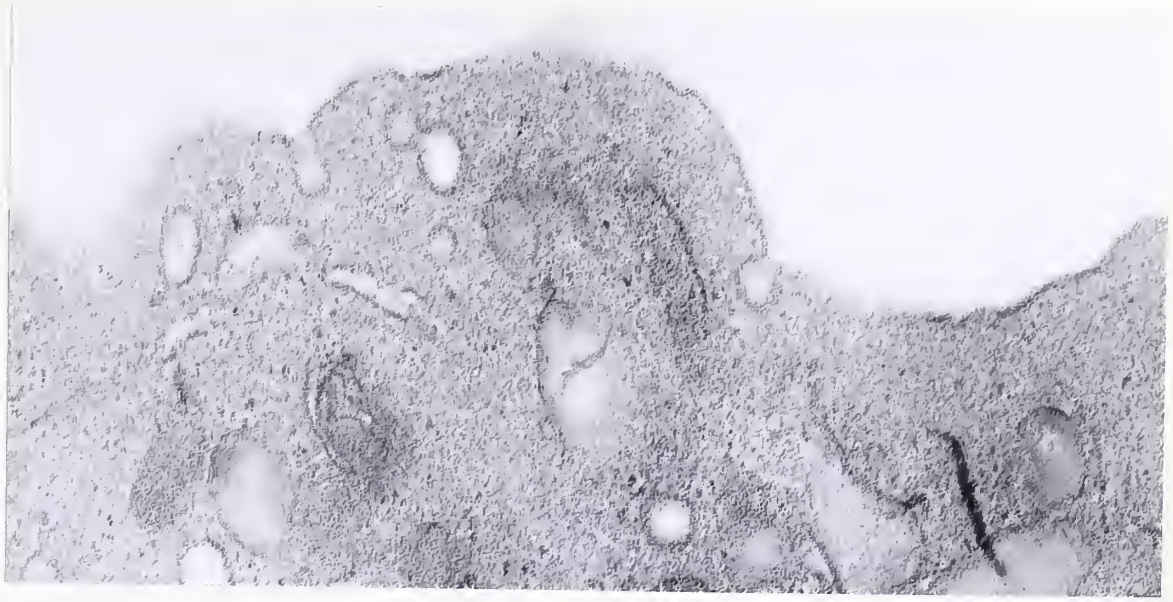
Figure 52 (middle)

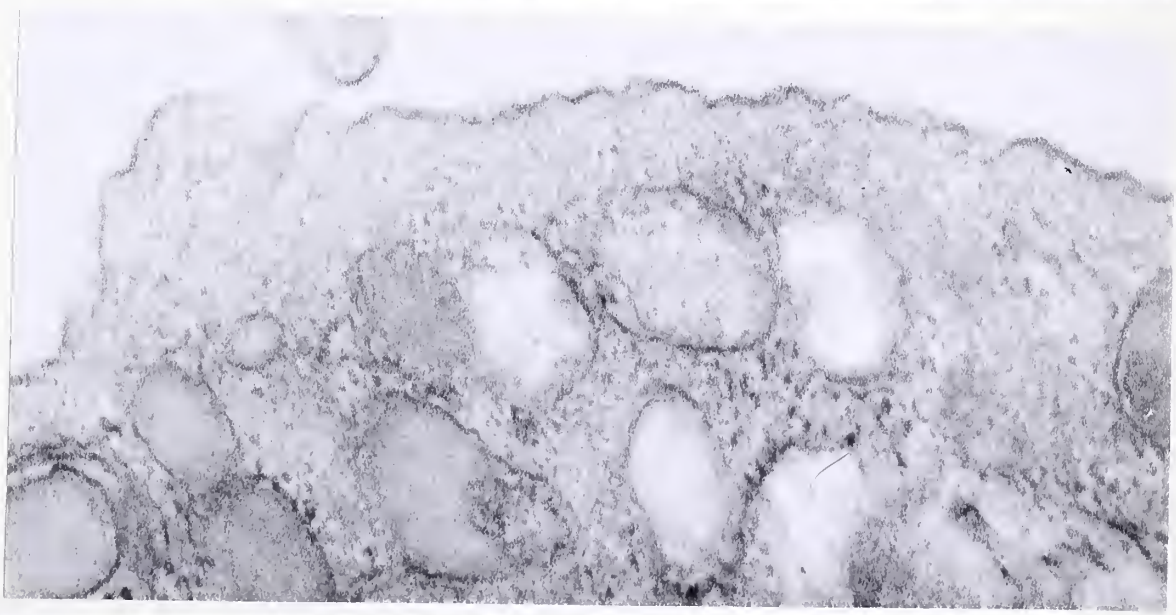
Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X116,000.

Figure 53 (bottom)

Fixation in glutaraldehyde and osmium tetroxide. Lead citrate stain. X116,000.

These high magnification views of the cell surface confirm the view expressed earlier: actinomycin blockade of EP release in PMN leucocytes treated with staphylococci does not block any of the morphological changes produced after incubation of normal cells with staphylococci.





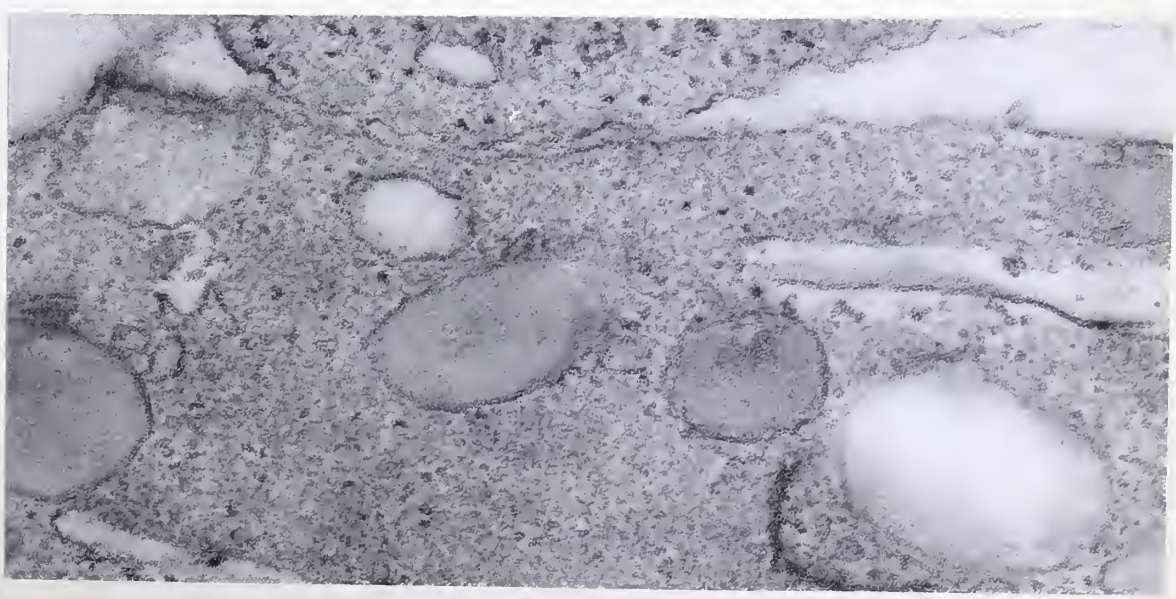


Figure 54

Human PMN leucocyte incubated with staphylococci and actinomycin (stained with PTA). A centriole from which microtubules radiate is seen in the left center of this micrograph. A description of the differences between this cell and actinomycin control cells appears on the next page. Fixation in glutaraldehyde. PTA stain. X36,000.

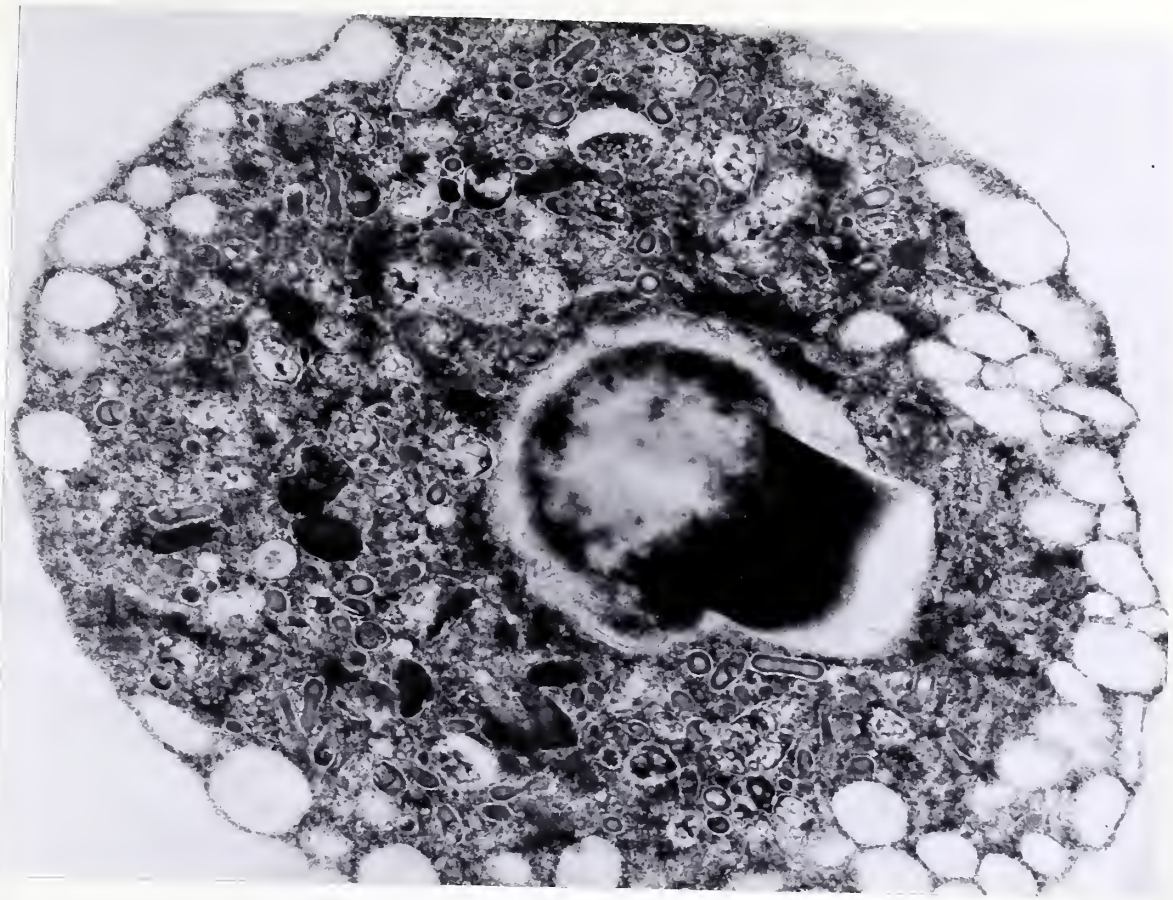
Figure 55 (above)

Human PMN leucocyte incubated with actinomycin (actinomycin control) (stained with PTA). The lucent vacuoles at the periphery of this cell, seen only in a few cells treated with actinomycin, probably represent a cytotoxic effect of actinomycin-D. Fixation in glutaraldehyde. PTA stain. X27,000.

Figure 56 (below)

Human PMN leucocyte incubated with staphylococci and actinomycin (stained with PTA). Peripheral lucent vacuoles are present in this cell as well. Fixation in glutaraldehyde. PTA stain. X25,000.

The actinomycin control cell appears similar to normal PMN leucocytes, with central clumped chromatin, a paucity of identifiable nuclear pores, a dense ground hyaloplasm, and few microtubules. The staph-actinomycin treated cell is similar to staph-treated cells, with peripherally distributed chromatin material, many nuclear pores, a relatively lucent ground hyaloplasm, and frequently seen centrioles and microtubules. Thus, the differences observed between these two populations are identical to those observed between staph-treated and control cells described earlier.



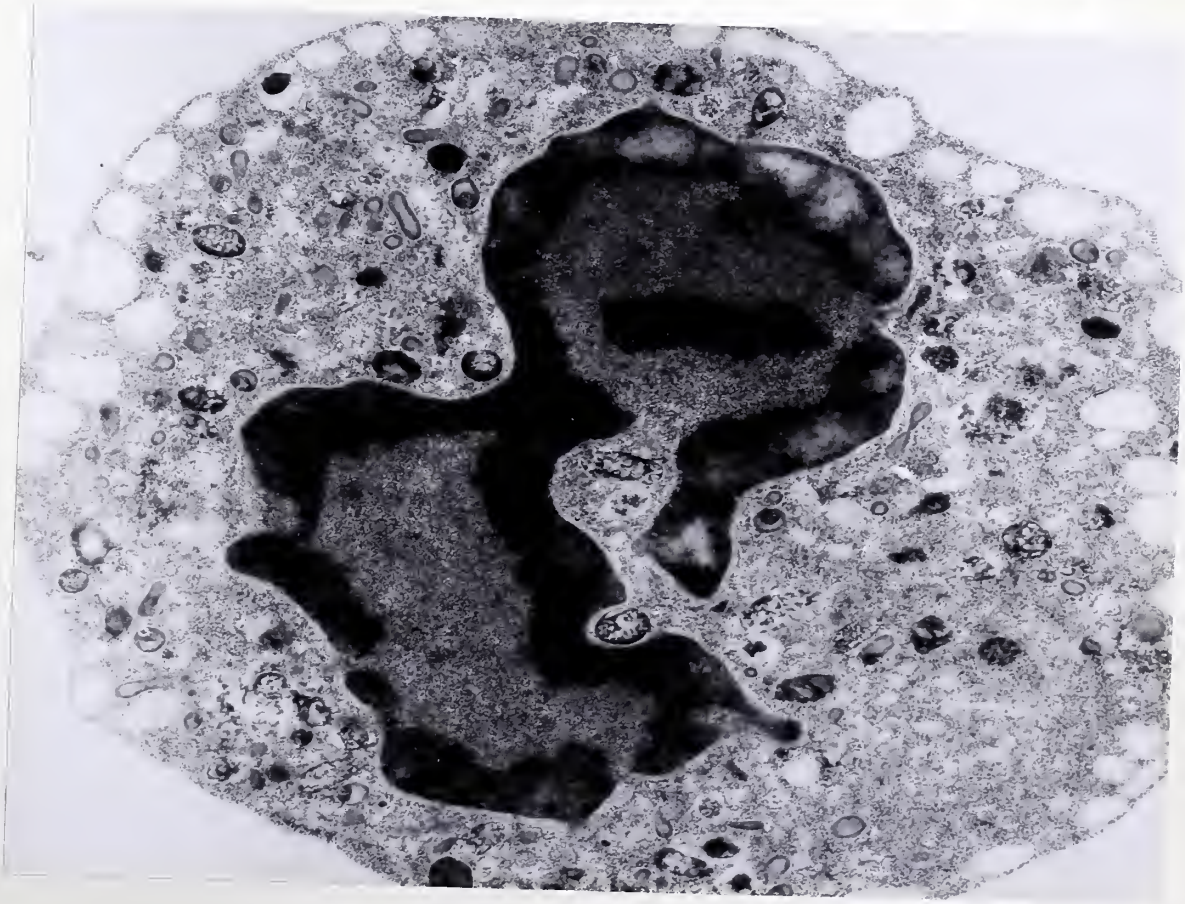


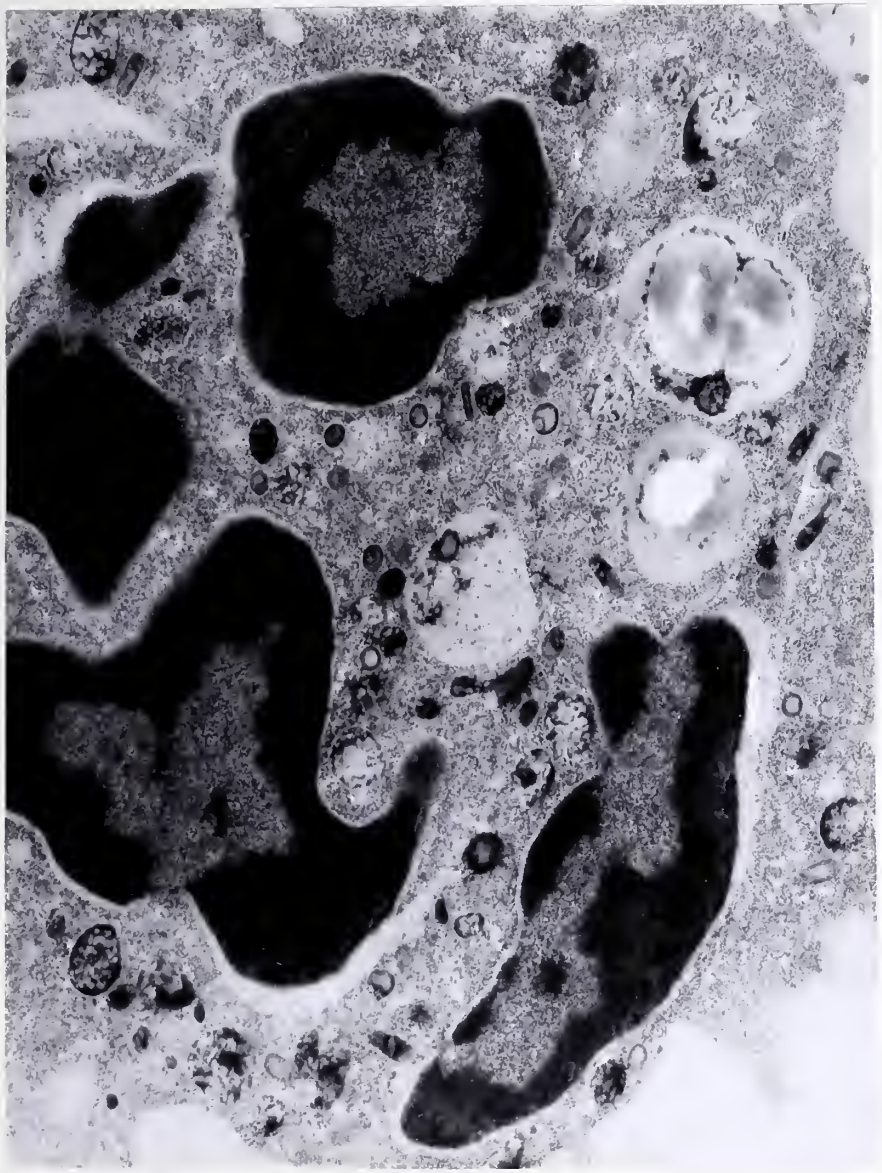
Figure 57 (above)

Human PMN leucocyte incubated with staphylococci (stained with PTA). Fixation in glutaraldehyde. PTA stain. X26,000.

Figure 58 (below)

Human PMN leucocyte incubated with staphylococci and actinomycin (stained with PTA). Fixation in glutaraldehyde. PTA stain. X29,000.

These two cells are not different in any respect except for a decreased number of lysosomes in the staph-actinomycin treated cell. As previously mentioned, this effect is seen in normal leucocytes treated with actinomycin, and represents an effect of actinomycin alone. Although actinomycin blocks EP release in leucocytes incubated with staphylococci, none of the morphologic changes seen after incubation with staphylococci is blocked. An identical conclusion was drawn earlier from observation of osmium-fixed PMN leucocytes.



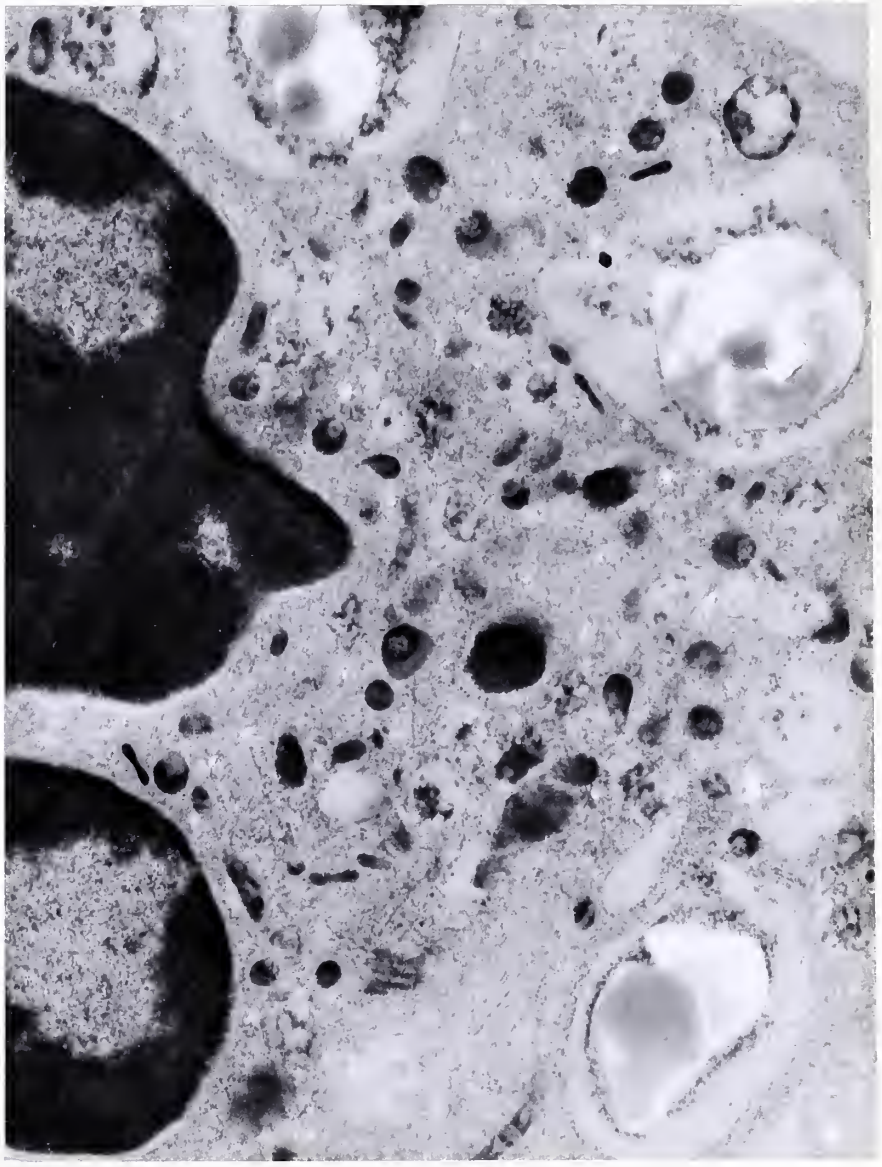


Figure 59 (top)

Control human PMN leucocyte. Fixation in glutaraldehyde. PTA stain. X106,000.

Figure 60 (above middle)

Human PMN leucocyte incubated with staphylococci. Fixation in glutaraldehyde. PTA stain. X106,000.

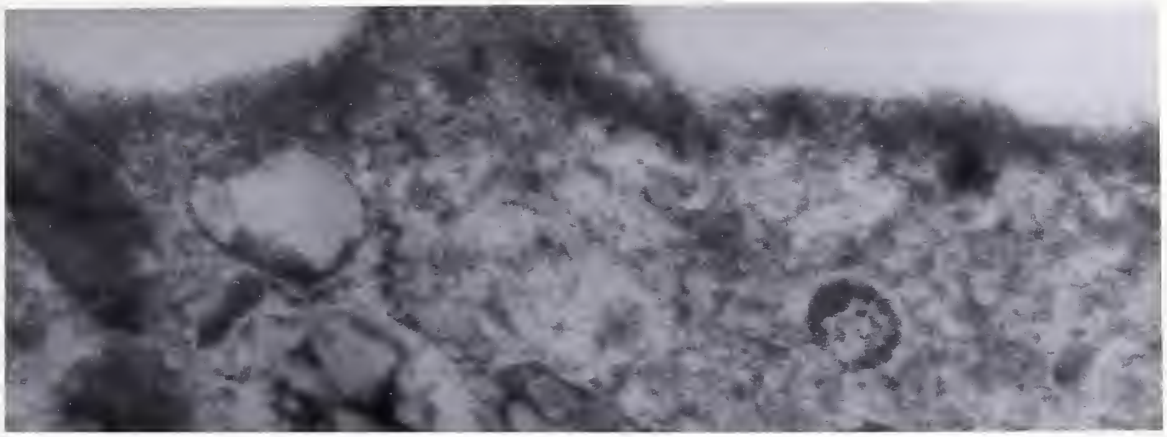
Figure 61 (below middle)

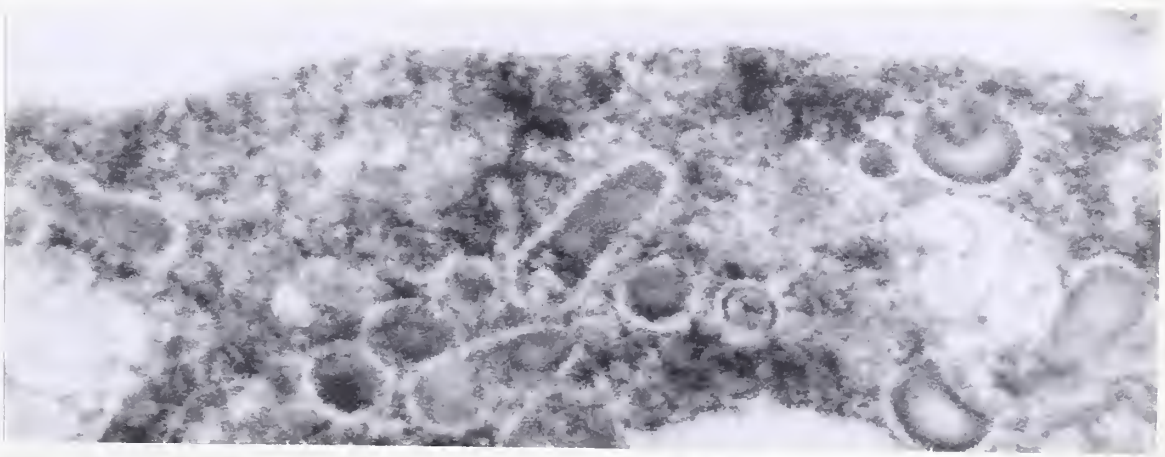
Human PMN leucocyte incubated with actinomycin (actinomycin control). Fixation in glutaraldehyde. PTA stain. X106,000.

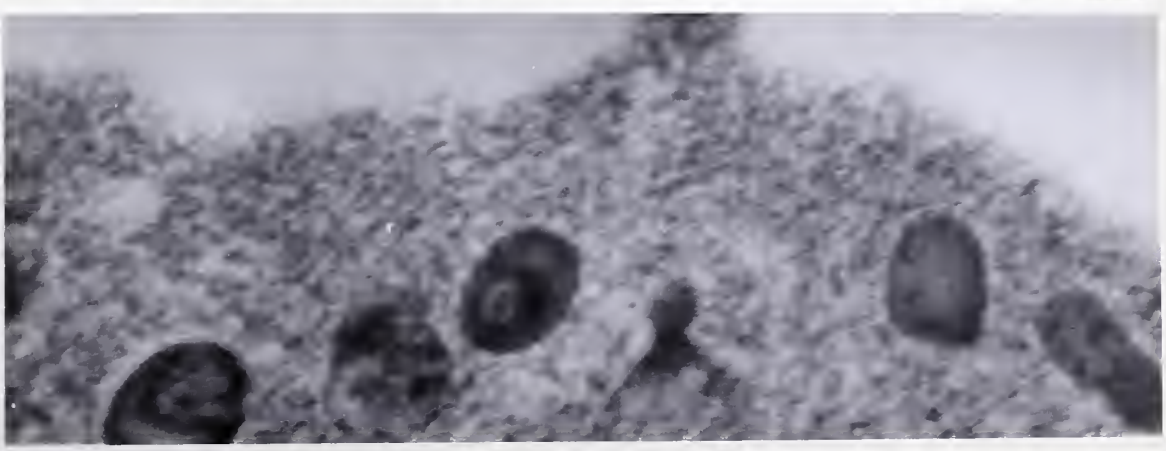
Figure 62 (bottom)

Human PMN leucocyte incubated with staphylococci and actinomycin. Fixation in glutaraldehyde. PTA stain. X106,000.

These high magnification micrographs were all taken at the cell surface of PMN leucocytes. In control and actinomycin control cells, the density of the ground hyaloplasm is evident, particularly subadjacent to the plasma membrane. Both staph-treated and staph-actinomycin treated cells have a lucent ground hyaloplasm without increased density at the cell surface. Comparisons among the four micrographs support the conclusion that morphologic changes produced by staphylococci are not blocked or reversed by the addition of actinomycin, even though EP release is blocked.







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